Home Office

Animals (Scientific Procedures) Act 1986

Non-technical summaries for project licences granted October – December 2024

Home Office

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1. Causes and consequences of corvid cognition

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Cognition, Behaviour, Evolution, Sociality, Corvids

Animal types	Life stages
Jackdaws (Corvus monedula)	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project will further our understanding of the evolution of intelligence using birds of the largebrained crow family as a model system. Using observations and behavioural experiments on wild jackdaws, we will determine how cognitive abilities allow individuals to respond to the challenges they face in their natural environment.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

1) Establishing how and why intelligence evolves is a central aim in biology, psychology and anthropology and to our understanding of human characteristics and our relationship with the rest of the natural world. This project will advance our understanding of the evolution of intelligence by studying wild corvids, birds of the crow family. Corvids are renowned for their large brains and sophisticated cognitive abilities, so by studying how they solve challenges in their natural environment we will better understand the evolution of intelligence in the animal kingdom.
2) The project will reveal how social bonds influence individual health, stress, cognitive performance and access to resources in natural populations. Understanding these

effects will contribute to the development of evidence-based interventions to promote animal welfare as well as human health and well-being.
3) Animals' cognitive abilities shape how they perceive and interact with their environment. Understanding the cognitive processes through which animals respond to changes in the environment is important for conservation and wildlife management as it will help us to predict the responses of animal populations to human-induced environmental change and to mitigate negative consequences.
4) The project will make important contributions to public engagement and science education. Findings will be disseminated widely through public workshops and exhibitions, tv, radio, press and social media, fostering understanding and appreciation of the scientific process and our native wildlife.

What outputs do you think you will see at the end of this project?

This overarching aim of this research is to advance basic knowledge by addressing fundamental questions about how and why intelligence evolves in nature. These questions are relevant to understanding why some animals have evolved to have evolved to have unusually large brains and sophisticated cognitive abilities. This is necessary both to determine why cognitive abilities vary across the animal kingdom (not only the corvids used in this project) and how human intelligence evolved. New information generated by the project will include datasets on wild jackdaw social structure, behaviour, cognition and welfare indicators (health and stress). Datasets will be made available on publicly accessible repositories such as Dryad and Figshare. Findings will be reported at scientific conferences and in publications detailing novel insights into cognitive evolution as well as novel methods for studying cognition in wild animals under natural conditions that can be widely used.

The information generated by the project will also provide new insights into the role of social relationships in mitigating stress and influence health. These insights may help to improve conditions for captive animals and develop guidelines to improve the welfare of wild animals that are impacted by human activities. Our work will also contribute to our understanding of the processes through which animals respond to changes in the environment. This will help us to understand, predict and mitigate the responses of animal populations to human-induced environmental change. Key outputs in this context will be publications and presentations of new discoveries at conferences and policy forums in animal conservation and welfare, talks for the general public and in global media.

Who or what will benefit from these outputs, and how?

1) The research community. The project’s principle outcomes will be publications published in academic journals. By revealing the cognitive abilities of wild corvids, their associated benefits and the interplay between cognition and social structure, this information will provide a major contribution to our understanding of cognitive evolution, a topic of central interest to biologists, psychologists and anthropologists. Our outputs will also be of benefit to biomedical and sociological research by revealing the influences of social bonds on stress and cognition.
2) Conservation and wildlife management. As cognition shapes animals’ responses to changing environments, cognitive research is increasingly recognised as a key component of conservation. Our outputs may therefore provide benefits for the development of effective conservation and wildlife management strategies. Specifically, the project will generate data on corvid foraging behaviour, social structure and social learning. Corvids are omnivorous and opportunistic and are commonly persecuted as agricultural pests in the UK, with millions of pounds spent annually on control and

deterrence schemes. However, data from other European countries suggest that corvids may provide important economic benefits by controlling populations of invertebrate agricultural pests (Hadjisterkotis, E. 2002 Z. Jagdwiss 49: 50-60). Moreover, as corvids are highly ecologically adaptable and behaviourally flexible, the efficacy of control measures is often limited. Detailed observations of foraging behaviour in this project will provide valuable empirical data with which to examine the agricultural impact of corvid populations, and hence evaluate the economic rationale for culling and other control measures. In addition, insights into the responses of wild corvids to novel problems and predators may assist in the development of efficient and humane deterrents if necessary. The work will also provide insights into the role of social learning in allowing novel information to spread through animal groups and in shaping group structure; issues that are increasingly recognised as being crucial to developing effective conservation strategies. Finally, our outputs will contribute to an understanding of why some animals can thrive in close proximity to humans whereas other species suffer population declines. This will contribute to conservationists' ability to predict and mitigate the responses of animal populations to environmental change.

3) Animal welfare. The structure of animal societies and individuals' positions within them are expected to be key determinants of responses to challenges in the environment. By revealing how social structure influences individuals' stress, health and access to information and resources in humandominated environments, the project will help to understand and mitigate negative responses to anthropogenic disturbance, maximise the efficacy of interventions designed to reduce human impacts and ultimately promote welfare in wild populations. By revealing processes at play in natural populations, outputs will also benefit the development of welfare measures for captive and managed animals.
4) Public understanding of science. As media coverage of my research attests, studies of animal intelligence capture the public imagination, promote appreciation of wildlife and provide an excellent means of scientific engagement with the wider community. Recent media coverage of my research group's work on corvids includes radio and tv documentaries in the UK and around the world and international press coverage in outlets including Science, National Geographic, New Scientist and the BBC. We have also run highly successful public science exhibitions on corvid cognition at schools and public events, which have attracted several thousand people. Thus, our work will provide educational benefits to school children, members of the local community and the broader public including national and international audiences.

How will you look to maximise the outputs of this work?

1) To ensure maximum accessibility by the research community, all publications will me made publicly available either in Open Access Journals or via the University of Exeter's Open Research Exeter (ORE) platform. All analysis code and data used in analyses will be made publicly available on an appropriate data sharing service (e.g. Dryad, GitHub).
2) Research findings will be presented to the scientific community and relevant stakeholders (e.g. conservation and animal welfare policy-makers) in conferences and seminars.
3) I am a member of the UN's Convention on the Conservation of Migratory Species (CMS) Working Group on Culture and Social Complexity, which provides an ideal forum for me to help apply insights from my research into the development of policy in animal conservation and wildlife management.
4) Research findings and protocols of relevance to animal welfare will be further disseminated via the Wild Animal Welfare initiative, which is a major funder of the work.

5) I have extensive media connections around the world and links with many local schools and community groups through my position as the Head of Outreach at the University of Exeter's Centre for Ecology and Conservation. I will harness these links to promote public understanding of science, reaching audiences of all ages at local, national and global levels through talks, workshops, science festivals and media.

Species and numbers of animals expected to be used

- Other birds: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Wild jackdaws (Corvus monedula) provide an ideal model system for studies of cognitive and social evolution. (1) Like other corvids, they are famed for their large brains and sophisticated cognitive abilities; (2) they live in complex societies containing long-term social bonds; (3) they take to nestboxes, allowing us to monitor reproductive success and (4) they have proven to be highly tractable for behavioural observations and field experiments.

This project will involve both nestlings and post-fledging juveniles and adults. Research with nestlings will be used to (i) monitor reproductive success (in terms of number and mass of nestlings that fledge the nest) and (ii) determine early life-conditions (e.g. stress states) affect the development of cognition and social bonds. Research on free-flying juveniles and adults will allow us to characterise the development of social behaviour and cognitive abilities and quantify the consequences of these abilities in terms of access to resources and reproductive success.

Typically, what will be done to an animal used in your project?

The great majority of the work is entirely non-invasive and poses no risk of adverse consequences. Nestboxes occupied by jackdaws will be monitored to record measures of reproductive success (clutch sizes, number and mass of nestlings). Behavioural observations rely primarily on video and automated monitoring to minimise disturbance caused by the presence of human observers. To enable individual identification and automated monitoring, jackdaws will be briefly trapped and fitted with a unique combination of coloured rings under licenses from the British Trust for Ornithology. To determine sex and quantify levels of parasites, stress hormones and diet, blood and feather samples will be taken from trapped birds (nestlings and free-flying adults) prior to being released back into the wild. A maximum of three small needle-prick blood samples may be taken from an individual within 12 minutes (maximum of five blood samples per individual per year).

What are the expected impacts and/or adverse effects for the animals during your project?

The only invasive procedures that will be carried out are the withdrawal of feathers (for hormone and diet analyses) and small blood samples (for analyses of hormones, parasites and determination of sex). These procedures are of mild severity and no adverse

consequences are anticipated (no adverse effects have occurred during the last 12 years of research using these procedures). Birds must be caught for sampling, which has the potential to cause stress. To minimise this risk, handling is conducted only by experienced, trained people (with appropriate BTO and Home Office personal licenses), handling time is kept to a minimum and birds are released immediately following sampling In the past 12 years of work at our study sites under previous PPLs we have developed rigorous protocols to minimise the risks of stress or injury , and have successfully trapped, ringed and sampled over 3000 birds with no adverse effects.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Maximum expected severity: MILD (100% of animals).

What will happen to animals used in this project?

- Set free

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There is no alternative to research on non-human animals if we are to understand of the factors favouring the evolution of intelligence. Without conducting research on wild animals, it is impossible to determine how animals respond to the specific challenges they encounter in their natural environments and thus the benefits that being intelligent may provide.

Which non-animal alternatives did you consider for use in this project?

The only possible alternative to the use of animals is to rely entirely on mathematical modelling. While this can have important value, it is not capable of addressing the research questions in this project.

Why were they not suitable?

Mathematical models can be useful in examining the theoretical efficacy of cognitive processing, but cannot explain how animals use their cognitive abilities to solve challenges under natural conditions. In this project, statistical analyses of field data (controlling for confounding factors) will determine (a) how corvids respond to challenges in their environment and (b) how these responses may provide benefits for health and reproductive success.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to

design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

All proposed experimental and observational protocols are based on previous successful research conducted by myself and others. Sample sizes for experiments will be determined by the balance between reducing the number of animals used and the need for large enough sample sizes to detect statistically meaningful effects. Specialised statistical techniques will allow any confounding factors to be accounted for, maximising the possibility of detecting meaningful patterns in the data.

As male and female corvids look alike, it is necessary to take small blood samples to determine their sex accurately by examining the sex chromosomes. Blood samples will also give accurate measures of current levels of stress hormones and parasites. Feathers provide a long-term measure of stress throughout development. The number of individuals sampled will depend on the rate of population growth, but based on our previous research numbers are likely to be fewer than 500 jackdaws each year to allow full coverage of nest-box users and their offspring.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All experimental designs are based on previous behavioural experiments that have been deployed successfully on wild corvids in my research group. Existing data from successful research over the last 12 years allows us to estimate the range of potential effects that may be detected in experiments, and thus the necessary sample sizes in order to detect statistically meaningful effects. Blood and feather sampling protocols have been refined to yield 90-100% accuracy in extracting necessary measures of individual characteristics.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use pilot studies in cases where existing data do not allow us to determine the range of likely responses to experimental stimuli in behavioural experiments. In addition, we will use analyses of simulated datasets to further optimise sample size decisions.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Corvids have large brains and possess sophisticated cognitive abilities, making them ideally suited for studies of the evolution of intelligence but most previous research has been conducted in captivity. The use of wild corvids represents a significant ethical

advance, given that captivity can cause substantial stress. The short-term, transient stress associated with capture and sampling is minimal compared to that experienced by such large-brained birds in captivity. The wild birds in our population are fitted with coloured leg rings for individual identification, under license from the BTO. Fitting leg rings does not produce detectable negative effects on survival in birds of this size.

With the exception of blood and feather sampling, the project is entirely non-invasive. Blood and feather samples are only used to confirm sex (necessary as jackdaws are sexually monomorphic) and quantify hormone and parasite levels and diet. Other sampling techniques (e.g. saliva or droppings) cannot produce data of sufficient quality and accuracy. However, we will use thermal imaging cameras to generate measures of stress non-invasively in freely interacting individuals without the need for capture. Cameras will be placed close to feeders or feeding tables, recording the eye temperature of visiting birds. Stress-induced vasoconstriction of surface blood vessels causes detectable reductions in eye temperature, so changes in eye temperature to infer changes in stress levels.

Blood sampling will be performed rapidly and to avoid unnecessary suffering. Small blood samples (< 100μl) will be taken from superficial blood vessels. The area will be swabbed with ethanol before sampling to prevent infection, and any bleeding will be controlled with gentle pressure. The birds are small enough to be gently restrained by hand and will be released immediately following sampling . Up to a maximum of three samples may be taken from an individual in a ten-minute period. Existing evidence shows handling and sampling does not affect nestling survival or provision of care by parents.

One feather will be removed rapidly immediately following blood sampling of jackdaws. Central tail feathers will be taken as they do not affect flying ability and will re-grow. The removal of a tail feather does not cause bleeding.

Why can’t you use animals that are less sentient?

The aim of the project is to shed light on the evolution of intelligence by studying the causes and consequences of intelligence the natural environment. It is therefore necessary to study animals known for their cognitive sophistication, at life stages where they can respond to in a natural way to their environments.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To avoid unnecessary suffering, the blood sampling procedure is completed within 12 minutes. Small blood samples (< 100μl) will be taken from superficial blood vessels. The area will be swabbed with ethanol before sampling to prevent infection, and any bleeding will be controlled with gentle pressure. The birds are small enough to be gently restrained by hand and will be released immediately following sampling. All staff involved will be trained and competent to handle birds with great care. Existing evidence shows handling and sampling does not affect nestling survival or provision of care by parents.

One feather will be removed rapidly immediately following blood sampling of jackdaws. Central tail feathers will be taken as they do not affect flying ability and will re-grow.

Adverse effects of these mild procedures are very unlikely, but the animals will be monitored carefully for any indicators of adverse effects during the procedure and post-release.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Association for the Study of Animal Behaviour: Guidelines for the ethical treatment of nonhuman animals in behavioural research and teaching. https://www.sciencedirect.com/science/article/pii/S0003347222002469

Redfern, C.P.F. & Clark, J.A. (2001) Ringers' Manual. British Trust for Ornithology.

Soulsbury, C., Gray, H., Smith, L., Braithwaite, V., Cotter, S., Elwood, R.W., Wilkinson, A. and Collins, L.M., 2020. The welfare and ethics of research involving wild animals: A primer. Methods in Ecology and Evolution, 11, 1164-1181.

Wild Animal Initiative: Guidelines on experimental design and ethical standards: https://www.wildanimalinitiative.org/rigor

Diehl, K. H., Hull, R., Morton, D., Pfister, R., Rabemampianina, Y., Smith, D., … Vorstenbosch, C. V. D. (2001). A good practice guide to the administration of substances and removal of blood, including routes and volumes. Journal of Applied Toxicology, 21, 15–23.

Miller, R., Schiestl, M. and Clayton, N.S., 2024. Corvids. The UFAW Handbook on the Care and Management of Laboratory and Other Research Animals, pp. 839-852.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I take active steps to implement up-to-date knowledge of advances in the 3Rs in my research. I follow updates and guidance on from the NC3Rs, the Association for the Study of Animal Behaviour and the British Trust for Ornithology, enabling me to follow any changes in guidance on the use of birds in behavioural research. I also attend workshops run by the Wild Animal Initiative (a foundation that promotes wild animal welfare) on best practice in field techniques and the measurement of stress in wild animals. My membership of my local animal ethics committee and the Animal Welfare Ethical Review Body further ensure that I remain continually engaged in current debates and guidance over practical techniques and debates concerning animal sentience and the 3Rs.

2. Brain fluid alterations in neurological disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Alzheimer's, Parkinson's, Neurodegenerative disease, Brain fluids, Cerebrospinal fluid

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To develop and characterise highly refined preclinical mouse models, to help us understand the role of brain fluid pathways in neurological diseases in humans, and test novel therapeutic targets aimed at curing these conditions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Neurodegenerative disease is the most common cause of death in England & Wales, and Scotland (slightly less common in Northern Ireland but still a leading cause of death). Neurodegenerative diseases include Alzheimer's disease, Parkinson's disease,

Huntington's disease, motor neuron disease (e.g., amyotrophic lateral sclerosis).

We have no cures for these conditions, and very few treatments, despite the massive impact they have on our lives. Further, we do not fully understand why these disorders occur, and what causes some individuals to become affected but not others. To aid and further our understanding of them, we require numerous different types of models, including cells, post-mortem human tissue, and animals. In this project, we will use mice to help us understand how and why these disorders develop, investigating the role that brain fluid pathways play in disease development.

What outputs do you think you will see at the end of this project?

At the end of our project, we aim to have acquired new information about the causes of neurological disorders of the brain and to have a thorough understanding of the involvement of brain fluid pathways in the development of these diseases. We aim to provide novel mechanistic insights into disease processes and identify aberrant pathways suitable for therapeutic targeting in the brain. Outputs may include disseminated publications, pilot proof-of-concept datasets for clinical translation of therapeutic candidates, and identification of novel compounds ready for further investigation and study.

All our studies have the eventual goal of developing therapeutic agents for the disorders modelled in mice, with knowledge disseminated to researchers to ensure that all therapeutic opportunities are exploited. As such, our close collaboration with clinicians (including neurologists and gerontologists) and basic scientists (including neurophysiologists and biochemists, neuropharmacologists, neuroimagers, behaviouralists) will ensure that we capitalise on all the information we gain from our use of animals in our research, and we constantly evaluate our findings in light of the human conditions we study.

Who or what will benefit from these outputs, and how?

Our research will have impact in the short-term, and in the longer term after its completion. In the short-term, the biomedical research community will be the main beneficiaries of our work, in that we will create new resources in the form of novel mouse models, disseminate new data on our specific disorders of interest, and importantly their underlying mechanisms of pathological development and novel insights into the roles of brain fluid pathways in their progression. Our use and development of highly refined mouse models will address specific research questions in the field, on the pathological causes of diseases characterised by neurodysfunction. Information gleaned from our in-depth phenotypic study of these animals will yield data on, for example, the affected cell types and aberrant molecular pathways in the brain, abnormalities detected through neuroimaging, changes in behaviour and key insights into the interactions between disease processes and brain fluid clearance pathways. This vital information resource will be used by us and others to help develop therapeutic strategies to tackle the raft of debilitating neurodysfunctional diseases in humans, as well as helping to develop biomarkers translatable to the clinic for earlier diagnosis and stratification of patient cohorts at risk of developing disease. Our hope is that one day, this will lead to an efficacious treatment and ultimately a cure for dementia.

How will you look to maximise the outputs of this work?

We believe that every mouse model should be studied by the widest group of experts possible, maximising wherever possible the data output from every animal life. We are a diverse group of researchers, experts in a plethora of research methods and techniques. Our aim is to provide deep and frequent longitudinal phenotypic information on each of the genetically altered mouse models of disease created in this project, characterising all aspects of brain health. Similarly, for other genetically altered animal lines e.g. those used to dissect pathways in the brain; deeply characterising their phenotype and utility as research tools. Some of the mouse strains we will be using in this project have already been thoroughly characterised, e.g. commonly used models of neurodysfunction. In this project we will use these models to study how alterations in brain fluid pathways are impacted in disease. Further, by experimentally altering brain fluid pathways in these mice we will learn how these affect disease development. Outside our immediate group of research scientists, we have a diverse network of collaborators, based in universities across the UK and further afield, and if unexpected phenotypic changes are observed, the insights of others working in a diverse array of specialities, e.g., heart, lungs, skin etc. will be sought, enabling holistic characterisation of novel mouse strains. All new knowledge obtained will be disseminated via publications, presentation of findings at research meetings, talks, and academic and lay data presentations and media outputs, maximising the outreach and spread of information we acquire throughout the course of the project. This is particularly pertinent when novel disease mechanisms have been identified, but target compounds have yet to be developed, facilitating their therapeutic targeting. By broadcasting our research outcomes to the widest possible scientific audience, we hope that our work will form the basis of new therapeutic development projects going forward. This equally applies to the publication/communication of negative results and instances in which our therapeutic interventions have been unsuccessful, as this data may provide the missing piece of the puzzle for researchers working in this field and others.

Species and numbers of animals expected to be used

- Mice: 6000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will work with mice because we can relatively easily manipulate the mouse genome to create animals that exactly mimic the genetics of people who have the disorders we work on. Also, until recently, it was very difficult to manipulate the genome of many species, other than mice, and so there are a wealth of different mouse strains that are deeply characterised and compatible with the newer mouse models we will work with. This will allow us to study the unique cross section and interactions between the development of neurodysfunction disorders, and brain fluid pathways, i.e., crossing animal models of Alzheimer's with animal models of impaired fluid clearance to determine if one intervention exacerbates the other. Further, it is now possible to use genetic alteration in mice as a research tool to understand neurodysfunction, e.g. use of animals in which specific pathways/cell types can be visualised in the brain to understand their function and/or involvement in disease development. We are interested in using mice at all life stages,

from the earliest stages of neurodevelopment right up until old age, where neurodegeneration is most prevalent, as depending on the model that we will use, we often will not know when neurodegeneration/dysfunction begins – as is the case with most human neurological disease. For example, it may be in the earliest stages of neurodevelopment, when the brains fluid filled compartments are being developed/formed, or it may be later in life, when age affects the ability of the brain to clear itself of toxic solutes.

Typically, what will be done to an animal used in your project?

We will study some animals in detail, to ascertain how the genetic alterations affect their brains as they age. This may include by techniques such as imaging, where the animal will be anesthetised and placed in an MRI scanner or PET scanner, providing us with images and measures of brain function with age/development. Other relevant techniques include behaviour, in which animals' memory or locomotion will be assessed in mazes or pieces of equipment designed to test aspects of their movement. Such tests might include the novel object recognition task, in which an animal is placed in a large open space and allowed to interact with two identical objects, e.g., water bottles. The test can then be repeated later (at different time intervals), switching out one of the water bottles for a new object, such as a tower of play blocks. By studying how the animal freely moves around this open space, and how much it interacts with the new object it has never seen before compared to the old object it should remember from its last session, we can get an idea of how intact the animal's recognition memory is. For other experiments, we may study how well the animal's brain clears away proteins, by giving a small injection of a known amount of a protein of interest (e.g., one of the proteins that builds up in the brain in Alzheimer's) into the mouse's brain. In situations like this, we might use imaging to watch the protein being cleared from the brain, or take samples of cerebrospinal fluid at certain points to measure how much of the protein has been cleared from the brain into the surrounding fluid. We might also alter the diet fed to the animals, to induce changes in their brains similar to what is seen with neurodysfunction; for example, giving mice food with more calcium in it to induce calcification of the brain's blood vessels similar to what we see in Alzheimer's. Lastly, we may want to look at the effects of a new drug we have developed. We might give young animals (that we have genetically engineered to develop aspects of Parkinson's disease) this drug over periods of time to see if it can delay/prevent symptom onset.

What are the expected impacts and/or adverse effects for the animals during your project?

Depending on the model of neurodysfunction, we expect to see different features in mice, most of which will be progressive with age and mild in severity. If left to age though, while we study the development of disease features in these animals, depending on whether additional procedures have been performed on the animals to test aspects of their brain fluid pathways or propensity to develop disease, some animals may experience moderately severe adverse effects. For example, we know that some procedures we may perform may lead to weight loss, depending on the genetic alteration the animal has, but generally this is insufficient to be a welfare concern. Similarly, if using transgenic mouse models, where the gene level is many times above what is normally expressed in patients, these animals may exhibit accelerated features of disease, and therefore may experience a moderate severity. These might include features such as altered behaviour and response to being handled or slowed weight gain/growth. For example, for mice modelling

movement disorders like Parkinson's disease, adverse effects might include altered movement and/or locomotion.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

In isolation, the majority of the genetic alterations used in this licence will produce only mild slowly developing symptoms, and hence their severity is not expected to exceed the mild limit. However, for some animals, i.e., those that are being aged to study the development of disease features, they may experience a moderate severity caused by their genetic alteration and development of pathology with time. Added to this, animals (including wildtype controls) which have had procedures performed on them to experimentally investigate disease development or features of brain fluid pathways may also experience moderate severity. In total, across both protocols, the expected proportion of animals in each severity category are below:

Subthreshold severity: 10%

Mild severity: 30%

Moderate severity: 60%

What will happen to animals used in this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The core aim of our research is to study brain fluid pathways and diseases of the nervous system. The brain is a highly complex organ, with countless cell types and cellular interactions. Dysfunction of this organ, leading to neurodysfunction and disease is an equivalently complex affair, involving unknown numbers of aberrant pathways and dysregulations. In order to study the effects of these dysfunctions, we can neither model behaviour other than with the whole animal, nor do we know yet about all of the cellular interaction and the effects these have on development, so we have to work on animals. Furthermore, structural organisation of the brain and it associated fluids and pathways leads to a biophysical scenario that cannot be modelled by any other means. Although the mouse lifespan is much shorter than that of humans, they nonetheless go through an ageing process that mirrors that in humans, and by careful experimental design we can model aspects of neurodegenerative disease in their brains. However, we note that mouse models are still models, and careful interpretation of research findings involving them is

required, despite their use in research being the most experimentally versatile and physiologically relevant option.

Which non-animal alternatives did you consider for use in this project?

The aim of our research is to relate our findings back to the human disorders that we are studying, i.e., neurodysfunctional diseases like Alzheimer's and Parkinson's disease. Therefore, in many situations, where possible, we will first verify and validate dysregulation of identified pathways/targets in human post-mortem diseased tissue prior to pursuing animal work. Such studies may involve in depth analysis of protein expression profiles aligning to the stages/anatomical locations of disease pathology in the brain. Although useful, these studies can only be used as validation alone, as dead tissue is limiting for facilitating study of the molecular and cellular interactions at play in complex disease scenarios. Cell culture systems represent an alternative system on which experimental interventions can be conducted. But for the majority of the targets related to brain fluid/clearance pathways, the nature of their intricate organisation and expression in the brain makes this extremely challenging and platforms such as cerebral organoids can be highly variable in their makeup. E.g., astrocytes in culture do not exhibit functionalised end feet as they do in vivo; importantly for our studies, protein expression in these end feet specifically facilitates glymphatic exchange of fluid from the perivascular space to the brain parenchyma. Lower vertebrates and invertebrates are often used in the field to understand the interplay between genetics and conserved molecular pathways. However, our combined goal of studying disease processes with brain fluid changes requires a systemic viewpoint, within which the complexities of the mammalian nervous system and unique brain anatomy can be studied.

Why were they not suitable?

Science has not yet advanced to the stage in which we can grow brain cultures of cells in a dish that reliably recapitulate the complexity of the brain in its structure, function, development or ageing in the body, let alone modelling the influences of hormones and metabolism, or brain fluid pathways on their function.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This is based on previous throughput in our research group and calculated based on the current and projected number of researchers working on this project over the course of its duration, and the required cohort sizes of mice to be statistically meaningful.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In all of our experiments we will use the minimum number of animals required to provide us with a statistically meaningful outcomes, based on a prior power calculation using expected affect sizes from the literature, or where available, preliminary datasets. We are careful to use appropriate control groups that are age-, sex-, and genetic background matched, in order to reduce cohort sizes, reusing datasets wherever possible. Further, the longitudinal use of novel imaging strategies in our research allows for repeated measures statistics in animal datasets, increasing the statistical power of the comparisons made. Prior to starting any animal experiments, the NC3Rs' Experimental Design Assistant will be used to aid and refine study designs.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We design breeding plans to work with the minimum number of mice that will give us the experimental and control genotypes needed for our experiments, undertaking small scale preliminary studies to determine effect size for power calculations wherever possible. In addition, resulting tissue samples from animal cohorts will be frozen/fixed, and banked within our laboratory to enable its use for future ex vivo analysis projects, and sharing with collaborators. This reduces the need to breed and age additional mouse cohorts, where in many cases samples have already been collected. Further, as part of standard laboratory practises in our group, all experimental designs, including data analysis plans will be approved by the PPL holder before experiments can start, and a protocol record for each experimental animal will be maintained by PIL holders (kept centrally by the PPL holder) which will include the description of the experimental steps, treatments and animal monitoring record. Archiving data like this allows for ease of retrieval as/when tissue samples are shared/re-used on other projects within our group for ex vivo analysis.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse is currently the only mammalian species that we can use to examine the full complement of parameters that are measurable in behavioural and physiological changes, cellular and molecular changes arising from neurodysfunctional disorder and altered brain fluid pathways, in concert with our ability to tailor the genome of these animals to maximise the information gained from each mouse. Our advanced use of imaging in our research, combined with behavioural assessment, means that we are constantly striving towards protocols that detect earlier changes, prior to major deterioration; such detection can only improve with time as we learn more about the mechanisms at play in the earliest stages of development. This is particularly important in our field of study of neurodegenerative diseases, where earlier detection/diagnosis may ease translation of therapeutic strategies into the clinic. We note that all our findings regarding early detection may be useful in

providing human biomarkers, which are badly needed for most neurodysfunctional diseases.

Why can't you use animals that are less sentient?

We are studying neurodysfunction in the mammalian nervous system, and while mice are different from humans at all levels, we are sufficiently close in evolution that basic mechanisms are almost always conserved, as are genes - which is not the case in non-mammals. Furthermore, the number of different mouse models available to the research community makes this an obvious choice of research species, allowing us to study the interaction of disease pathways of neurodysfunction with fluid pathways in the brain, building on decades of work already achieved using mouse models in the field.

Other vertebrates may be used to understand some of the biology of the disorders we study but are not the best at recapitulating pathology such as, for example, behaviour. Similarly, while many aspects of the central nervous system are conserved from humans to invertebrates and may help us understand disruption to these tissues, organisms such as fish or flies do not harbour the complexity and intricacies present in the mammalian nervous system, particularly when it comes to the study of brain fluid systems, and mammalian models better recapitulate complex pathological changes in disease. For example, invertebrate models often carry far fewer genes than mammals and do not have the paralogues or even orthologues found in the mammalian genome, making modelling of some disorders impossible. Genetic systems in fish may be relevant for some disorders but the manipulation of the fish genome is nowhere near as sophisticated as that for mouse and cannot currently give us the refined models we work with for human disease, and again, fish may not have the specific genes we wish to investigate.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For all tests it is important that mice have no additional stress, therefore mice are handled by experienced operatives only, calmly, and are habituated to testing rooms as well as arenas as far as possible. Animals will be group housed with access to clean bedding and nesting material, and will have access to additional environmental enrichment in their cages. Tunnel handling or cupping are used for all mice. Mice undergoing behavioural tests in mazes or arenas, or on specific pieces of apparatus, will be removed from their home cages for the minimum time needed to gather meaningful data. Mice undergoing phenotyping tests have increased monitoring and are removed from tests if they appear to be suffering from an adverse stress reaction, or other unexpected adverse effects of the tests.

Animals undergoing surgical procedures will receive peri-operative analgesia. Animals will be monitored intensely after surgical procedures and after substance administration to check for clinical signs. Directly after surgery, animals will be constantly observed until they have recovered from the anaesthesia, and then monitored hourly within working hours and first thing the next morning. Added to this, mice will receive routine health checks and weighed daily for a week post-surgery, to ensure full recovery. If any animal shows signs of stress, ill-health, or failure to thrive post-surgery, it will be culled. But in our experience, this is a very rare event. The procedures within this project are run routinely in our lab, and in our experience animals recover rapidly after surgery/imaging sessions.

Experimental pipelines are designed with thought given to the overall, cumulative experience of the mouse and the number and type of tests being conducted on it. With the constant support of the NACWOs and NVSs we will strive to continually refine our experimental mouse workflows over the duration of this project.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Routes and volumes for administration of substances are taken from the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group's 'Refining Procedures for the Administration of Substances' report. All surgical procedures will be conducted following LASA guidelines for aseptic techniques. All animal experiments will be designed with the assistance of the NC3Rs Experimental Design Assistant, and we will adhere to ARRIVE2 guidelines in publication and communication of experimental outputs. Our laboratory, and our institution conform to the highest level of quality and welfare control on all fronts, including husbandry, phenotyping and administrative processes. Standard operating procedures for most tests we will perform are already generated, using data and expertise from experts in each field.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We regularly receive information and updates regarding the 3Rs within our institution and attend 3Rs events, maintaining close relationships with our local NC3Rs representatives. When we develop 3Rs advances ourselves, we present these at 3Rs meetings, for example the PPL holder recently gave a talk regarding updates on the use of non-invasive imaging in mouse models of Alzheimer's disease.

With the support of our Named Veterinary Surgeons (NVSs) and Named Animal Care and Welfare Officers (NACWOs), we strive for constant refinement of our work using animals and will continue to do so throughout the duration of this project.

3. Pharmacokinetics of Agrochemicals

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
    - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph

Key words

Pharmacokinetics, Metabolism, Tolerability, Agriculture, Veterinary

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Provide a service to assess the tolerability, metabolism and pharmacokinetics of new chemical substances being developed by clients to improve yield of food crops, animal welfare and prevent disease. Data produced will support the progression of novel agrochemicals with the aim of applying for regulatory approval of these substances.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

With current trends for increases in the global population, and the impact of global warming on crop production, the evolution of animal and plant diseases and infestations, there is a need to improve the productivity of farming activities to enable sufficient food production. The substances under investigation in the studies authorised by the licence will be directed towards this overall aim. Such substances will treat plant infections and infestations or be used in farm animals to treat parasitic infections.

Due to the quantities of these substances used in agriculture there is a strict regulatory requirement to ensure that they are safe from a human, animal, and environmental, perspective. This requirement includes understanding effects caused by both short-term exposure and also if small levels of substances persist in the body. Persistence is particularly important for food-producing animals as the substance will ultimately reach the human population with the potential to put health at risk.

The decision-making studies to determine safety are long-term toxicology studies that would be conducted under separate authorities, but a critical step towards performing these studies is to understand how the substances are taken up into, changed by and removed from the body.

Many aspects of the behaviour of a substance can be predicted by computer modelling approaches, and further understood by studying its physical properties and behaviour in cell-based experiments. These results allow the initial development of the substance by for example changing its structure or the material used to dissolve it.

However, these approaches currently are not fully predictive of how the substance will behave in, and be changed by, the body, with all the complex organ systems interacting. The use of animal models helps provide this understanding ensuring that only the substances with the least likelihood of problems of persistence in the body are taken forward testing in toxicology studies.

The tolerability studies performed on this licence are normally the very first time a substance will be administered into a living vertebrate animal and will investigate how the animals respond to dose levels required for the subsequent studies performed as part of this licence. These studies check for any obvious adverse reactions to ensure that a very early decision can be made on continuing to work with a substance whilst using a very small number of animals.

What outputs do you think you will see at the end of this project?

The studies performed under this licence will contribute to the design of regulatory testing strategies for new substances designed to improve agricultural output and will build on the information produced in studies performed without using animals.
The animal studies performed under this licence will provide information on how a novel substance interacts in a complex organism with similar biological processes to humans. This will help scientists to understand whether to progress with developing their substance, or using the evidence of absorption, spread, and persistence within the body, plan improvements to the substance or design the following studies to understand the safety of the substance.

Where significant results are not subject to confidentiality agreements they will be communicated more widely at scientific meetings or published in peer reviewed journals.

Who or what will benefit from these outputs, and how?

Clients will be able to make progression decisions to be able to better target their financial resources on to the substances with the highest likelihood of being approved by regulators.

In stopping the progression of projects with little chance of success due to adverse pharmacokinetics, metabolism or tolerability will also mean that animals will not be used in safety studies.

In the long term the successful identification of safe and improved chemical substances to increase yield of food will improve and protect the food supply globally.

How will you look to maximise the outputs of this work?

Where there may be broader interest in an animal modelling approach, and if the studies are not subject to confidentiality agreements, these will be published or shared at relevant conferences. Refinements to techniques will be shared with others working in the field via individual contacts made by the establishment's Named Persons

Clients and collaborating partners will be encouraged to publish all results in journals or share at relevant conferences.

Species and numbers of animals expected to be used

- Mice: 1050
- Rats: 5100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

These animal studies serve as a bridge between computer or cell-based experiments and safety assessment studies. The similarity and differences of the rodent models used in this licence are well (although not completely) understood when compared to higher species used for safety assessment and humans, allowing modelling approaches based on the rodent data to determine initial dose rates for safety assessment studies and assist with interpreting those results for regulatory submissions.

Adult animals will be used as these are representative of animals used in safety assessment studies.

The animals used in this project will be rats and mice as these have been demonstrated to provide reliable information that enables decisions to be made on the progression of

agrochemicals.

Typically, what will be done to an animal used in your project?

In a tolerability study small numbers of animals will be given substances at dose administration rates similar to those that will be required for initial pharmacokinetic studies. The route of administration will generally be injection into blood vessels, or delivered directly into the stomach via a stomach tube, but may rarely be on to the skin.

Normally a single dose will be administered, but in some studies, there will be repeated administrations. The animals will then be regularly observed for up to 48 hours to identify any symptoms that may mean the substance is unsuitable for further studies. Depending on the project requirements, there may be higher or lower doses administered to subsequent animals until a clear understanding of the tolerability is established.

Routine pharmacokinetic and metabolism studies are normally conducted in rats and mice, as these species have been identified as being suitable models to provide data for further studies in higher species and man.

A typical study in the rat would involve the surgical implantation of a catheter into the jugular vein, which allows high quality samples to be taken without disturbing the animals within the cage. Following recovery for several days, administration of a substance is then performed, generally by injection into blood vessels in one group and delivered directly into the stomach of a second group to enable comparisons to be made between these two common routes of administration. Normally only a single dose will be administered by each route, however in some studies there will be repeated administrations. At time points relevant to the proposed clinical use of the substance samples of blood will be withdrawn from the jugular vein catheter, generally for up to 48 hours following dosing. In some cases the two routes of administration may be performed in the same animal several days apart to be able to compare the response within the same individual which reduces the total number of animals used.

For routine pharmacokinetic studies in mice, a typical study would involve the administration of a substance generally by injection into blood vessels in one group of mice and delivered directly into the stomach of a second group to enable comparisons to be made between these two common routes of administration. For substances that will be handled in a farm environment it may also be necessary to measure how much material is absorbed through the skin by placing the substance on to the shaved skin of rats or mice.

Normally only a single dose will be administered by each route, however in some studies there will be repeated administrations. At time points relevant to the proposed use of the substance on the farm microsamples of blood will be withdrawn from the tail vein (normally up to 8 samples in 24 hours). If it is critical to understand the level of the substance in a particular tissue it may be necessary to euthanise mice at each timepoint to sample the tissues for analysis.

Where animals have undergone jugular vein cannulation surgery, and the Named Veterinary Surgeon is happy that it is appropriate from severity, scientific and animal health aspects, the animals may be used for further pharmacokinetic studies. Once recovery from surgery is complete, the limited disturbance to the animals for the pharmacokinetic studies mean this approach reduces the overall suffering involved by

reducing the numbers of animals undergoing surgery.

To understand how the substance is removed from the body it may be necessary to place the animals in a cage with a grid floor over an apparatus that separately collects the urine and faeces (a metabolic cage) by themselves for up to 24 hours (rarely up to 48 hours), which helps identify whether the kidneys or liver are involved in this process.

To enable pharmacokinetic studies to be performed on animal tissues, it is often necessary to use freshly prepared (where immortal cell cultures are not suitable or available and commercially available tissue may not be suitable due to delays in delivery reducing effectiveness). In this case animals are anaesthetised and blood collected from the heart. The animal is then euthanised, and other relevant tissues (normally liver, kidney, heart and muscles) are taken for use in the test system

What are the expected impacts and/or adverse effects for the animals during your project?

In tolerability studies it is theoretically possible to see a wide and unpredictable range of impacts due to this being the first administration to an animal, however screening in non-animal systems prior to first dose in animals means severe adverse effects are very rarely seen. Typically at the doses and routes being used the animal sometime may exhibit subdued behaviour, pain at injection site, altered breathing patterns, weight loss (normally caused by loss of appetite). These are generally short-term effects as most substances are cleared from the body within a few hours, but rarely may be present for the duration of the study (48 hours in most cases) and will not result in euthanasia if considered of mild severity.

Similar mild adverse effects may be seen following substance administration in the other studies performed under this licence and will be controlled by careful choice of dose level following tolerability assessment, and the application of humane end points if they are greater than expected.

Administration of substances and blood sampling from the tail vein causes brief stress and pain due to handling and needle insertion. These are controlled by skilled handling and minimising the numbers of administration and sampling events.
Surgical cannulation of blood vessels will cause pain that is controlled by using pain-relieving drugs: generally, this lasts for 48 hours, but the animals are closely monitored in case further doses are required.

Being placed in a metabolic cage for collection of urine and faces is mildly stressful but will routinely be limited to a maximum of 24 hours (rarely for a maximum of 48 hours <5%).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice - 50% Mild and 50% Moderate

Rats - 20% Mild and 80% Moderate

What will happen to animals used in this project?

- Killed
- Kept alive at the establishment for non-regulated purposes or possible reuse

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Many of the initial studies performed to understand the pharmacokinetics and metabolism of substances used in agriculture now take place using computer simulations, assessments of the physical properties of the substance, then in enzyme- cell- and plant-based experiments. Using the results generated by these methods many substances are not taken forward into animal experiments.

However, even with these advances, non-animal approaches still do not allow the interactions of the substance with the body to be assessed in a way that reflects the full complexity of an integrated mammalian system.

This project aims to provide the data from a complex, integrated organism to allow decisions to be made on whether to progress substances used in agriculture to the next stage of development.

Which non-animal alternatives did you consider for use in this project?

Some or all of computer modelling, physical property assessment and enzyme- and cell-based approaches will be used prior to undertaking studies on protected animals to minimise the number of studies and potential impact on the animals.

Prior to performing animal studies clients will be asked to provide information on the work undertaken with approaches not using protected animals and an outline of literature reviewed searching for alternative approaches. It would be expected that non-animal approaches will have been undertaken to have confidence that the novel agrochemical is likely to have suitable pharmacokinetics for progression to toxicology studies. These tests will reduce the numbers of toxicology studies performed and increase the likelihood of those completed to deliver meaningful results.

Why were they not suitable?

There are no non-animal alternatives that can currently replicate the full complexity of absorption, distribution and metabolism of a substance in the mammalian body. Now and for the foreseeable future there will need to be animal experiments performed to perform initial investigations into pharmacokinetics as part of the process of bridging from non-animal studies to safety assessment studies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated numbers are based upon likely study numbers proposed by a major client that is seeking to perform their pharmacokinetic studies at this Establishment. In addition, other clients are anticipated to commission work to be performed under the authority of this licence.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Consultation with biostatisticians has indicated that 3 animals per group gives an effective assessment of inter-animal variability whilst still providing decision making data. Lower numbers are considered inappropriate due to inadequate understanding of variability in responses.

The extensive use of modelling within project teams has minimised initial pharmacokinetic studies to a single administration normally via two routes of administration (intravenous and oral): this allows decisions on progression to be made and studies investigating the toxicology of the substance to be accurately designed.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where appropriate and accepted by the client, the use of cross-over designs (when investigating the same substance) or re-use of surgically prepared animals (to investigate different substances) will reduce the number of animals undergoing surgical procedures.

Where a project permits, cassette dosing studies (where several low doses of substances are administered simultaneously), may be performed. This will enable multiple compounds to be screened simultaneously, thereby reducing the total number of animals used.

When animals are euthanised to provide tissues, other users within the company are offered the remaining tissues to ensure maximal usage.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why

these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Jugular vein cannulated rats allow for central blood samples (considered to be the most appropriate samples) to be taken with minimal disturbance to the rat. The use of skin buttons for exteriorisation in longer term animals, although requiring more invasive surgery than the use of exteriorised catheters with a pin port, does allow for group housing. Use of buprenorphine jelly for analgesia, with regular clinical assessments to determine whether additional doses are required minimises the post-surgical pain.

For non-surgical pharmacokinetic models, the use of micro sampling methods together with removal of scab rather than repeat insertion of needles reduces the pain associated with sampling. Exposure to a warm environment to ensure dilation of the tail veins is reduced to the minimum required to help reduce the stress associated with this procedure. Tail vein cannulation reduces the number of needle insertions required where larger sample volumes are needed.

Why can’t you use animals that are less sentient?

Currently pharmacokinetic modelling in less sentient species such as zebrafish or fruit flies does not provide information that can be used for progressing to humans without the need for mammalian studies. These studies are useful only when working on the species in question and would simply represent an additional step in the process rather than a decision-making study.

These simple animals do not provide sufficient data to enable the development of a thorough understanding of the effects of the substance in a fully integrated organism, which allows the complex interaction of the novel agrochemical with many body systems to be assessed prior to moving on the human clinical studies.

Most studies will be investigating the progression of process that occur over more than one day, so the use of terminal anaesthesia is not appropriate in most cases.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

On site, working proactively with the AWERB, NVS and NACWO, there is a culture of constant improvement to animal care, control of adverse effects, performance of procedures and study design.

Scoring systems are used to identify early intervention and end points in studies, pain-relieving drugs are used when there is concern an animal is suffering, and monitoring is performed as often as required, including throughout the night.
Further refinement to technical procedures and housing are implemented when they are shown to be beneficial for the animals and will not reduce the quality of the scientific outputs.

When a refinement to an established model is identified from the scientific literature and proposed for use under this licence, a small number of pilot studies will be performed using standard compounds to ensure the model delivers high quality scientific data whilst allowing the development of adverse event controls and scoring systems that may be able

to be used to reduce the severity experienced by the animals.

When placed in metabolic cages, enrichment items (such as shelters) that do not interfere with sample collection will be placed in the cage.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

In addition to Home Office and EU guidance documents, relevant best practice guidance will be sourced from the NC3Rs (e.g. ARRIVE Guidelines, blood sampling, experimental design), NORECOPA (e.g. PREPARE guidelines), LASA (e.g. blood sampling, drug administration, aseptic surgery) RSPCA and model-specific publications.

Specific regulatory guidelines that are applicable to the proposed work include:

OECD (2010), Test No. 417: Toxicokinetics, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, https://doi.org/10.1787/9789264070882-en.

US Environmental Protection Agency: Health Effects Test Guidelines OPPTS 870.7485 Metabolism and Pharmacokinetics, August 1998.

Japan MAFF Test Guideline 2-4-2 Metabolism in Livestock (2014).

UK HSE. REACH etc. (Amendment) Regulations 2021

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

AWERB and the Named Persons routinely circulate information about the 3Rs and identify opportunities for enhancements during their routine rounds.

On a monthly basis the PPL Holder receives and reviews automated literature alerts on animal models and journals relevant to the project licence.

When a new animal model is proposed, a thorough literature review is performed to determine the most scientifically relevant approach whilst causing the least harm, and a new automated alert generated.

In addition to conference attendance, webinars and discussion groups are participated in by the PPL Holder and scientific staff.

4. Increasing the success of glaucoma filtration surgery

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Glaucoma, Fibrosis, Therapeutics, Implants, Devices

Animal types	Life stages
Mice	adult
Rats	adult
Rabbits	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The main objective of this project is to develop new treatments to minimise scarring in glaucoma. This will include new wound healing modulating drugs, new devices in glaucoma surgery and improved drug delivery techniques in the eye.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Glaucoma is an eye condition where the optic nerve is damaged by the pressure of the fluid inside the eye. There is a large unmet clinical need being addressed in this project, with glaucoma being the leading cause of irreversible blindness and affecting over 70 million people worldwide. Glaucoma filtration surgery is the mainstay of surgical treatment in glaucoma, but it fails primarily due to scarring in 50% of patients after 5 years of follow-up. The toxic anti-cancer drug, mitomycin-C, is the current gold standard to prevent scarring in glaucoma filtration surgery, but it leads to severe adverse side effects such as tissue damage and severe infection.

This project aims to develop new treatments to prevent scarring in glaucoma patients. In addition, new improved animal models will increase our understanding of the disease mechanisms of complex wound healing in glaucoma surgery. This project will also help us to identify new therapeutic targets and to develop new drug delivery techniques and devices that could benefit millions of glaucoma patients in both developed and developing countries.

What outputs do you think you will see at the end of this project?

1. Improved drugs to prevent scarring after glaucoma filtration surgery in adults and children. This will lead to more successful surgical outcomes in glaucoma.

2. New glaucoma drainage devices that allow for better flow control and therefore prevent potentially blinding post-operative complications. Similar to traditional glaucoma filtration surgery, new glaucoma drainage devices will primarily fail due to scarring. New glaucoma devices thus need to be combined with improved anti-scarring drugs to increase the surgical outcomes in glaucoma.

3. Refined slow release drug delivery methods to improve wound healing post-operatively and the surgical outcomes in glaucoma.

4. Increased understanding of the disease mechanisms of complex wound healing in the eye and identification of new therapeutic targets and biomarkers.

Who or what will benefit from these outputs, and how?

1. Patients (next 5-10 years)

Our main impact goal is to increase the success rate of glaucoma surgery and to decrease preventable blindness from glaucoma. New anti-scarring drugs, drainage devices and improved drug delivery methods will lead to reduced complication rates and hospital visits, thereby significantly improving the quality of life of glaucoma patients and their families.

2. Health Professionals (next 5-10 years)

A new anti-scarring treatment will help ophthalmologists to better treat their patients, by increasing the surgical success of glaucoma surgery and without exposing them to the risk of toxic anti-cancer drugs like mitomycin-C.

3. Socioeconomic and Decision-makers (next 5-10 years)

A reduction in the side effects of the current toxic anti-cancer drugs will lead to less post-surgical complications, including reduced hospital visits and rate of re-operations, thereby saving costs for healthcare providers (NHS cost savings through post-surgical complications of over £40,000 per patient). On average, a patient with complications is hospitalised 1.2 - 3.8 days longer than those with no complications, costing an average of £1,711 - 4,225 including drugs, clinical costs and rooms.

4. Academic and Scientists (short-term, next few years)

The results and databases will be published in open-access journals so that the research will also benefit the scientific community.

How will you look to maximise the outputs of this work?

I will publish our methods and results in open-access journals so that the research will benefit the scientific community. Fibrosis is a multisystemic disease and I am part of the FLARRE (Inflammation, Tissue Repair, Scarring & Fibrotic Diseases) consortium. I will present our results at the annual FLARRE meetings and at national and international conferences. I will also set up collaborations with other researchers working on fibrosis in the eye and other parts of the body.

Species and numbers of animals expected to be used

- Mice: 1000
- Rats: 1000
- Rabbits: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Where possible, mice or rats (adults) will be used in this project but due to their small eye size, certain techniques of glaucoma surgery and examination are difficult, and in such cases, rabbits (adults) will be used instead. The rabbit, rat and mice models are validated models of eye scarring that have previously helped in the translation of the anti-cancer drug, mitomycin-C, in glaucoma surgery.

Typically, what will be done to an animal used in your project?

PROTOCOL 1: WOUND HEALING STUDIES

1. Eye examination (All animals)

All animals will be examined after surgery. The eye pressure will also be measured using a

portable pressure-measuring instrument.

2. Glaucoma filtration surgery (All animals)

Surgery will be performed on one eye and the other eye will be used as a control non-operated eye. The animals will undergo surgery under general anaesthesia.

3. Administration of anti-scarring drugs (Optional)

Anti-scarring drugs will be applied using different drug delivery techniques and to specific parts of the eye.

4. Eye fluid sampling (Optional)

Under general anaesthesia, a small volume of aqueous fluid (fluid in the front of the eye) and/or vitreous humour (jelly-like fluid at the back of the eye) will be withdrawn using a very fine needle.

5. Blood sampling (Optional)

A small volume of blood will be withdrawn from the ear (rabbit) or from the tail (mouse/rat) using a fine needle.

6. Killing (All animals)

The animals will be killed at various intervals up to six months after surgery by a schedule 1 method.

PROTOCOL 2: GLAUCOMA DRAINAGE DEVICES

1. Ocular examination (All animals)

All animals will be examined daily after surgery. The eye pressure will also be measured using a portable pressure-measuring instrument.

2. Surgery for optimisation of a new glaucoma drainage device (All animals)

Surgery will be performed on one eye and the other eye will be used as a control non-operated eye. Surgery may include insertion of a drainage tube or implant or material into the eye under general anaesthesia.

3. Administration of anti-scarring drugs (Optional)

Anti-scarring drugs will be applied using different drug delivery techniques and to specific parts of the eye.

4. Eye fluid sampling (Optional)

Under general anaesthesia, a small volume of aqueous fluid (fluid in the front of the eye) and/or vitreous humour (jelly-like fluid at the back of the eye) will be withdrawn using a very fine needle.

5. Blood sampling (Optional)

A small volume of blood will be withdrawn from the ear (rabbit) or from the tail (mouse/rat) using a fine needle.

6. Killing (All animals)

The animals will be killed at various intervals up to six months after surgery by a schedule 1 method.

What are the expected impacts and/or adverse effects for the animals during your project?

1. Pain

Analgesia will always be given to all animals before surgery, during surgery and for as long as required after surgery. Artificial tear eye drops will also be used for eye lubrication and comfort when necessary. If any animal shows signs of pain (e.g. failure to eat, failure to groom, pawing the eye), analgesia will be given immediately. If these signs of pain persist after analgesia is given or if any animal appears to be suffering significant distress, the animal will be killed by a schedule 1 method. Any animal showing failure to eat will be given diet gel or food supplements. If no improvement after 1 week, the animal will be killed by a schedule 1 method.

2. Rise in eye pressure

If there is an increase in eye pressure after any surgery, the eye pressure will be monitored every 2 hours and treated with anti-glaucoma eye drops. If the eye pressure is measured above 40 mmHg, this would be considered as the end point and the animal will be killed by a schedule 1 method.

3. Low eye pressure

A transient low eye pressure may be observed after surgery and this usually resolves without further intervention. However, if there is persistent low eye pressure (less than 5 mmHg) after 24 hours, the animal will be killed by a schedule 1 method.

4. Eye inflammation (uveitis)

Mild inflammation may be observed after surgery but usually gets better by itself. The animals will be treated with steroid eye drops and killed by a schedule 1 method if not responding or worsening.

5. Infection in the eye

Good clean technique will be used during surgery and antibiotic ointment will be applied to the eye at the end of surgery. The animals will be monitored for signs of infection including eye redness, lid swelling and discharge. If infection develops in the eye, the animal will be killed by a schedule 1 method.

6. Subconjunctival bleeding and bleeding inside the eye

Bleeding inside the eye during surgery usually gets better by itself. If the bleeding still persists, the animal will be killed by a schedule 1 method.

7. Lens opacity and lens dislocation

Careful surgical technique will reduce the risk. No action will be taken if lens opacity occurs. In the event of lens dislocation, the animal will be killed by a schedule 1 method.

8. Discomfort from pellets of material placed in the subconjunctival space (uncommon with current drugs and small volume of pellet)

If the drug or pellet causes significant discomfort or is coming out, the animal will be killed by a schedule 1 method.

9. Discomfort from injection

Local anaesthetic eye drops should provide adequate anaesthesia. If new drugs are used which cause more discomfort than current drugs (unlikely) at the time of injection, general anaesthesia may be considered.

10. Adverse response to general anaesthetic

The general anaesthetic regime we use is efficient and adjusted according to weight, but individual animal's responses to anaesthesia can be variable. Rarely, an animal may fail to recover from anaesthesia. Any animal that fails to fully recover will be killed by a schedule 1 method.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Moderate severity for all animals (100%) undergoing experimental glaucoma surgery.

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Although we make extensive use of *in vitro* (laboratory-based, cell culture) models in this project, there is currently no effective way to model pathological scarring in glaucoma surgery outside the living animal. The new treatments must be able to prevent scarring *in vivo* (in living animals) to be successful in the clinic and the use of animals in this project is

therefore essential to achieve this aim.

Which non-animal alternatives did you consider for use in this project?

In vitro (laboratory-based, cell culture) models, e.g. collagen contraction assays, cell adhesion studies, cell viability assays. *Ex vivo* eye tissue models will also be used as non-animal alternatives.

Why were they not suitable?

Due to the complex nature of the wound healing process, *in vitro* studies alone only provide small clues to what may happen in the entire organism. To this end, only *in vivo* (in living animals) models can provide the accurate and complete results we require before taking our work forward towards human clinical trials.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

During this project, we will use the minimum number of animals to achieve our scientific aims. We have estimated the numbers of animals that we will use based on the experimental needs of the research and on any publication justification from published literature. Based on previous studies, we will use 6 animals per experimental group as this is the minimum number of animals required to show significant differences.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will use the NC3Rs' Experimental Design Assistant to ensure that we use the minimum number of animals to meet the scientific objectives of the project. We also collaborate with statisticians and they will help with the sample size calculation and experimental design.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Our decision to use wound modulating agents or devices will be guided by extensive *in vitro* (laboratory-based, cell culture) studies. We will also conduct small pilot studies on 2-3 animals to give us an indication as to the efficacy of the treatment, therefore aiding in the design of the experiments to use the minimum number of animals. We might also share tissue with other researchers and collaborators.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative

care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The animal model used has been refined and optimised in my hands to deliver the research with good success rates. We will minimise animal suffering with the use of anaesthesia during each surgical intervention. We will always give analgesia after surgery. We will also limit the number of times an animal undergoes a drug dosing and animals will be kept for the minimum duration required. The rabbit, rat and mouse models are all validated models of glaucoma surgery. The rabbit eye is more similar in size to a human eye and will be used for surgical procedures where the rat and mouse eyes are too small.

Why can't you use animals that are less sentient?

The rabbit, rat and mouse models are all validated models of glaucoma surgery. The rabbit eye is more similar in size to a human eye and will be used for surgical procedures where the rat and mouse eyes are too small. A zebrafish eye is very different from a human eye and it would not be possible to perform glaucoma surgery in zebrafish eyes.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To minimise suffering, we will administer appropriate anaesthesia for every surgical intervention. Analgesia will always be given to all animals before surgery, during surgery and for as long as required after surgery. We will also monitor the animals carefully in the post-operative period and any animal deemed to be suffering will be removed from the study by a Schedule 1 method.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the NC3Rs guidelines on the 3Rs (Replacement, Refinement, Reduction) and the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines, and the Association of Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I am subscribed to the NC3Rs newsletter and get monthly updates about new advances in the 3Rs. I will contact the NC3Rs Regional Programme Manager for advice during the project.

5. Zebrafish models of neurological disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

motor disorders, neurological disease, characterisation, therapeutic development

Animal types	Life stages
Zebra fish (Danio rerio)	adult, juvenile, neonate, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the project is to address the lack of treatments for neurological diseases. We hope to address this by generating zebrafish disease models, characterising them, and using them to investigate new therapies.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The work is important because there is currently a lack of effective treatments for the vast majority of neurological diseases.

What outputs do you think you will see at the end of this project?

We expect to publish new findings in relation to the underlying biology of neurological diseases, and the identification of novel therapeutic approaches for these diseases.

In addition work conducted during this project will provide important pilot data to enable us to secure future funding.

Who or what will benefit from these outputs, and how?

We hope that our findings will be of relevance to a number of stakeholders. For example, other researchers in the field of neurological disease, patients and families afflicted by the diseases we study, and pharmaceutical companies interested in utilising our models and therapeutic approaches in order to formally progress our ideas into a clinical setting. In addition we hope the findings will enable us to secure future funding for research.

How will you look to maximise the outputs of this work?

We disseminate our findings through scientific and lay presentations. We collaborate closely with other investigators where it is mutually beneficial, for example working with clinical geneticists to generate disease models.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 15000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We use zebrafish because they offer several technical advantages compared to alternative species such as mice for our experiments.

Fish are vertebrates (i.e. they have a spinal cord) so represent a simple yet appropriate model for studying human neurological diseases.

The majority of our work involves the use of non-regulated embryonic life stages.

Typically, what will be done to an animal used in your project?

Genetically modified zebrafish will be used primarily for breeding and maintenance. Most research will use embryos at non-regulated stages. In some cases, zebrafish >5dpf will be used for behavioural analysis to assess neurological phenotypes.

Zebrafish will be used to generate genetic models of human neurological disease. Mutations will be introduced into the genome to mimic mutations found in human patients (100 procedures over 3 months).

As stated above, most research using the genetically modified zebrafish bred under this license will use life stages up to 5dpf. However, we will also characterise these zebrafish using protocols included in this license (e.g. imaging to characterise behaviour, or treatments to induce a phenotype), in order to determine whether they develop a version of the human disease at the molecular, physiological and pathological level (500 procedures over 1-2 years).

In addition we will use approaches to cause or accelerate neurological disease. These approaches include various exposures, such as a irradiation to cause damage to DNA, or exposure to various environmental agents that have previously been linked to the development of neurological disease in patients.

At the molecular level we will look for biochemical changes associated with the mutation in man (e.g. changes in mitochondrial form and function). At the physiological level we will look for alterations in behaviour. At the pathological level we will look for pathology reported in patients, e.g. death of specific neurons and protein aggregation.

If we are able to show that these zebrafish model the human disease then we will use them to help to find new treatments for the human disease. This will usually involve treating the fish with drugs, typically delivered in their diet or in the aquarium water, and then seeing if drug-treated fish show any improvement compared to fish receiving placebo (200 procedures over 1-2 years).

Further analysis of these drug trials will typically involve pathological and biochemical confirmation of the effects of the drug (no additional procedures, over 1 year).

What are the expected impacts and/or adverse effects for the animals during your project?

The most likely adverse events will be the development of symptoms of neurological disease, such as impairments in their swimming or behaviour. We judge these to be moderate severity, since they do not cause signs of distress. However the impairments may last several months, and be progressive in nature (in most cases reflecting the human disease). Animals will be killed humanely to provide tissue samples for our research.

In addition to the expected effects above, because we are using approaches such as chemical or irradiation exposure, fish may experience toxicity associated with this. For example chemicals linked to the development of neurological disease are likely to have other unpredictable effects, and irradiation may cause the development of tumours.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All experiments will use zebrafish.

mild: 80%

moderate: 20%

severe: 0%

What will happen to animals used in this project?

- Used in other projects
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To characterise molecular mechanisms of neurological diseases we must perform some experiments at the level of the whole organism. We use cells and tissue samples where it is possible to do so, but ultimately we need to understand how neurons die in their natural context.

Which non-animal alternatives did you consider for use in this project?

Human cells expressing disease-associated mutations.

Primary cultures of rodent-derived embryonic neurons.

Why were they not suitable?

Neurons are highly specialised cells, which interact with a wide variety of other cell types both inside and outside the brain and spinal cord. For example a motor neuron in the lower spinal cord (small of the back) can send processes, over a metre long, out to muscles in the foot and in so doing makes unique and intimate interactions with at least four different cell types. Each interaction has its own complicated chemical and physical signals. Such complexity is impossible to replicate in culture systems

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We will use appropriately controlled experiments. In the case of genetically modified fish, controls will be wild type siblings. In the case of chemical or other treatments we will use vehicle or untreated fish. For environmental toxins we will use a range of doses based on relevant published data, and tissue levels may be confirmed using techniques such as mass spectrometry.

Variability is inevitable in outbred zebrafish stains. For some experimental approaches we have years of experience, and can determine group sizes on the basis of this experience. For other approaches pilot studies using small groups are performed to quantify variability in experimental readouts, and then determine an appropriate number of animals to generate satisfactory data.

Fish are randomised for therapeutic, chemical and irradiation treatments. Blinding is unnecessary for the majority of behavioural tests which use unbiased and objective automated tracking techniques.

For the majority of experiments multiple readouts are obtained from individual animals, such as critical swimming speed, behavioural flexibility, working memory. This maximises the amount of data we obtain using the fewest possible animals.

For general breeding, we maintain up to 40 fish (typically 20 male and 20 female) of a particular genotype ant any time. This is sufficient for routine embryo production.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

As stated previously most experiments are conducted on fish <5.2 days old, which reduces the number of animals used (i.e. we just need to maintain breeders).

For experiments with protected stages, we consult experienced statisticians for assistance with design of new experimental approaches, analysis of data, and feed this forward into future experimental planning. For the majority of studies with protected stages in this license we base experimental design on established protocols in the lab.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Zebrafish are highly efficient breeders, so we are able to obtain large numbers of embryos <5.2dpf from a small number of parents. Where feasible we use approaches to genotype embryos at nonprotected stages, in order to be able to raise on the particular genetic We will use pilot studies to optimise the number of animals used in this project. We will share tissue where it is feasible to do so.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use zebrafish models of human neurological disease because they offer several technical advantages compared to alternative species such as mice for our experiments.

Fish are vertebrates (i.e. they have a spinal cord) so represent a simple yet appropriate model for studying human neurological diseases.

The models themselves are generally mild, but occasionally moderate severity. On balance, this outweighs the unmet need for scientific advance in order to identify therapeutics.

Why can’t you use animals that are less sentient?

The majority of animals used under this license will be embryos <5.2 days old. These are not considered protected animals for the purposes of ASPA, thus we already use the least sentient model. Where we do use life stages >5.2 days old we believe that zebrafish are less sentient than mice, which would be the most suitable alternative model system.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Sometimes, for example after fin biopsy for genotyping, we require short periods of single housing of zebrafish, and where we don't think this will jeopardise the welfare of the genetically modified fish we will use companion animals.

We use close monitoring of adult zebrafish disease models, including when needed the use of score sheets to monitor levels of distress. In the event that genetically altered zebrafish shows any distress, for example caused by abnormal swimming, this allows us to implement a humane endpoint.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We use sources listed on the NC3Rs Zebrafish welfare website (NC3Rs, ZHA, ZFIN). We also consult NACWOs for advice.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will follow the relevant literature for experimental design, use twitter to find new information, attend relevant meetings (eg FELASA).

To implement changes I will work with my research group to pilot new approaches where we believe these may improve 3Rs aspects of our work.

6. Tumour cells and their microenvironment in cancer progression and metastasis.

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Cancer, Metastasis, Microenvironment, Ageing, Therapy

Animal types	Life stages
Mice	adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to understand how tumours grow and spread to other sites in the body, with a particular focus on how the tumour cells interact with the cells around them and how changes that occur in the surrounding cells during events such as ageing can affect tumour cell behaviour. By doing so this project aims to identify new treatments and test the ability of these treatments at preventing the progression and spread of tumours, ultimately uncovering new potential treatments for patients.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The vast majority of cancer-related deaths occur due to secondary disease, where the cancer has spread to other sites in the body. Treatment options for patients with secondary cancer is limited. Therefore, there is an urgent need to better understand the mechanisms behind the progression and spread of the tumours, to improve treatment options for these patients.

What outputs do you think you will see at the end of this project?

It is expected that this project will advance our knowledge of the mechanisms behind the progression and spread of the cancer, and particularly how the tumour cells interact with other cell types in their vicinity to facilitate their spread and growth. Additionally, it will improve our understanding of how events such as ageing or age-related conditions such as tissue scarring (known as fibrosis) can lead to changes in the surrounding cells and environment that can then impact tumour cell behaviour and spread to secondary sites. It will also result in characterisation of novel ways of modelling secondary disease in mice. Finally, it will uncover novel treatment strategies to prevent or treat secondary disease and provide evidence of their anti-tumour activity in mouse models. It is anticipated that data generated from this project will result in publications which includes the depositing of large collections of data (datasets) on freely available repositories. Data from this project will also be presented to the scientific community at conferences to share the findings as widely as possible with other researchers.

Who or what will benefit from these outputs, and how?

The outputs of this project (the advancement of knowledge of the mechanisms of cancer progression and spread and treatment approaches, large collections of data (datasets) on ageing and cancer spread and characterisation of new models) will benefit other researchers studying how tumours progress and spread. Data on the impact of ageing and fibrosis (tissue damage/scarring) will also benefit the wider, non-cancer researcher community. Due to the nature of the models, and particularly the ageing models, and the need to robustly validate the findings before publication these outputs may not be fully realised until the end of this project, although myself and others in my group will present the data at meetings and conferences throughout the duration of the project. Ultimately, the aim is that the data generated from studies in mice in this research project will form the foundation for follow-up studies that are focussed on developing therapies against secondary cancer and may result in direct patient benefit.

How will you look to maximise the outputs of this work?

In order to maximise the outputs of this work I will need to make the data and findings as widely available as possible. To do this I will publish my findings in open-access journals. Datasets will be made freely available by submission to the online data repositories upon acceptance for publication. Throughout the course of this project myself or members of my group will present the findings at scientific conferences and meetings, and establish collaborations with other researchers to facilitate prompt dissemination of new knowledge and support the progression of the research project. Any novel mammary tumour cell lines used to establish the models in this study will be made available upon reasonable request. Unpublished data will be made available at appropriate points in the research programme through collaborations. I aim to publish any useful datasets generated or unsuccessful

approaches that may not be relevant for inclusion in research publications, by uploading open access publishing platforms.

Species and numbers of animals expected to be used

- Mice: 2700

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The spread of cancer to a secondary site in the body is a multi-step process, which includes the tumour cell invading out of the primary tumour and into the bloodstream, surviving in the circulation, exiting the bloodstream and invading into secondary site, and surviving and growing at that site. At every step the tumour cell is interacting with other cell types and the local environment. Therefore, to understand how a tumour cell spreads to another site and grows there we need to carry out studies at the whole-body level. Transplantation of tumour cells into mice is a simple and efficient method for investigating cancer growth and spread (metastasis). Moreover, to assess the effect of treatments at limiting the growth and spread of the cancer we need to perform experiments in mice to assess the full therapeutic effect of these agents in the presence of all cell types. We need to use mice and cannot use other model organisms, for example zebra fish, because mice are mammals and are biologically very similar to humans: most genes in mice share functions with genes in humans; humans and mice have very comparable immune systems; the development of diseases such as cancer in mice mimics very closely how the disease develops in humans. We will be performing these experiments in adult mice - both young adult mice (typically 2-3 months old), as used in the majority of preclinical mouse studies, and aged mice (>12 months). The impact of ageing on tumour growth, spread and response to treatment has been a severely neglected area of research, despite the fact that the majority of cancers are diagnosed in patients in the later decades of their lives.

Typically, what will be done to an animal used in your project?

The typical mouse will be injected with tumour cells, either at the primary site (for example in the mammary gland to model breast cancer growth and spread of the cancer), which may involve surgical removal to mimic what happens in the clinic and allow outgrowth of secondary cancer, or by other routes: e.g., injected into the tail-vein to allow spread of the cancer to the lungs and other secondary sites, or into the leg bone, liver or abdominal cavity to study growth of secondary cancer at these sites; or the mouse will receive a therapy that will lead to local tissue scarring (fibrosis). The mice may receive treatments prior or following tumour cell injection or fibrosis-induction to assess the effect of the treatment on the growth and the spread of the cancer and fibrotic/scarring response, respectively.

Experiments with hormone-dependent cancer cell lines may require implantation of a slow-release hormone pellet under the skin prior to injection of the cancer cells. Surgical procedures will be carried according to current best practices and will receive appropriate pain relief before and after surgery to minimise pain. Mice may be imaged up to twice per

week to monitor tumour growth and/or may have blood samples taken during the course of the experiment. Young and aged mice will be used. In some incidences local tissue scarring (fibrosis) will be induced as above in tumour-bearing animals to assess the impact of a fibrotic environment on cancer growth and spread. Typical experiments last 3-8 weeks.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice will experience mild, transient pain and no lasting harm from the administration of substances using standard injection routes. Mice may experience some longer-lasting mild pain or discomfort due to the growth or cancer in the body or local fibrotic (scarring) responses. Wherever possible tumour growth in animals will be monitored by gentle palpation, calliper measurement or by non-invasive imaging to ensure the tumour burden in the animals does not become too substantial. To study and treat secondary cancer (metastasis) large primary tumours will be required to ensure that there has been enough time for the cancer cells to go through all steps in the process and the primary tumour has fully established and developed an adequate blood supply to support escape, seeding and growth of the cancer cells at other sites in the body. These primary tumours will grow under the skin or in the mammary gland and be easily accessible to monitor and measure at all times. Mice receiving treatments to induce fibrosis may gradually develop signs of inflammation (body's immune response to injury) and swelling or stiffness of the local tissue and may experience some weight loss. Body weights, condition of the animals and any signs of pain or distress will be monitored for all mice. We will ensure they do not exhibit continued signs of pain (pain at an intensity that requires analgesic treatment that cannot be alleviated within 48 hours) or weight loss (>20% of animal's average stable adult body weight). Mice receiving surgery for example to implant a pellet under the skin that can release the hormone slowly, to remove the primary tumour that is growing under the skin, or to inject cells into the liver or the bone marrow, are expected to recover quickly and will be given painkillers and post-operative care and receive enhanced monitoring.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice: expected severities are mild (10%) and moderate (90%). All protocols are moderate but some non-tumour-bearing controls or control (vehicle, for example saline)-treated mice for fibrosis experiments are expected to be mild severity.

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To assess how tumour cells and the non-tumour cells of the environment interact, it is essential to carry out studies at the whole-body level. Transplantation of tumour cells into mice is a simple and efficient method for investigating cancer growth and spread (metastasis), and the effect of any therapeutic agent on limiting this.

Which non-animal alternatives did you consider for use in this project?

I am experienced in several advanced in vitro (experiments performed in dishes or on plates in the lab) techniques which are superior to culturing tumour cells on 2D plastic. These include growing cancer cells in 3D spheroids ('mini-tumours') or organoids (3D cultures that mimic the tissue structure found in the body) and co-cultures (cultures of mixes of different cell types) which can be grown on different matrices (material to mimic the tissue between the cells in the body). Where possible, I perform experiments using these 3D cultures, as part of my project (for example to perform some intricate mechanistic studies based on in vivo findings) and in all instances I consider whether any of these in vitro approaches could be used instead of performing the experiments in mice. I perform regular searches on the NAT (non-animal technologies) database for any technologies that can be used to study metastasis (search term metastasis, selecting cell culture, tissue models, organoids, spheroids as methods). This brings up results including spheroid cultures, co-cultures etc, which are systems I employ currently. However, none allow the modelling of the whole tissue microenvironment with all the cell types and matrix (material found between the cells) components as found in tissues, and these cultures continue to be of limited use when studying spread of cancer (metastasis) as they only model one step in the metastatic process. Similar, when considering techniques to employ to assess the efficacy, dosage and tolerability of therapies I perform searches on drug treatments and drug toxicity using cell culture methods. When a new potential therapy is identified I test it on cells in these cultures to confirm it is hitting the target and having an anti-cancer effect but there is no way of confirming whether it works as a drug treatment while having limited side-effects or associated toxicities without performing these experiments at a whole-body level in mice.

Why were they not suitable?

Despite advances in in vitro techniques, they are limited, somewhat artificial and cannot replicate the complex environment that exits in the primary tumours and secondary sites, for example the immune system plays a fundamental role in controlling the growth and spread of the cancer cells and this complex system cannot be replicated outside the body. Therefore, the use of mice still provides the most physiologically relevant system. The tumour and fibrosis models that we use in these studies are widely recognised and published as being suitably refined to minimise the level of distress and suffering to the animal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

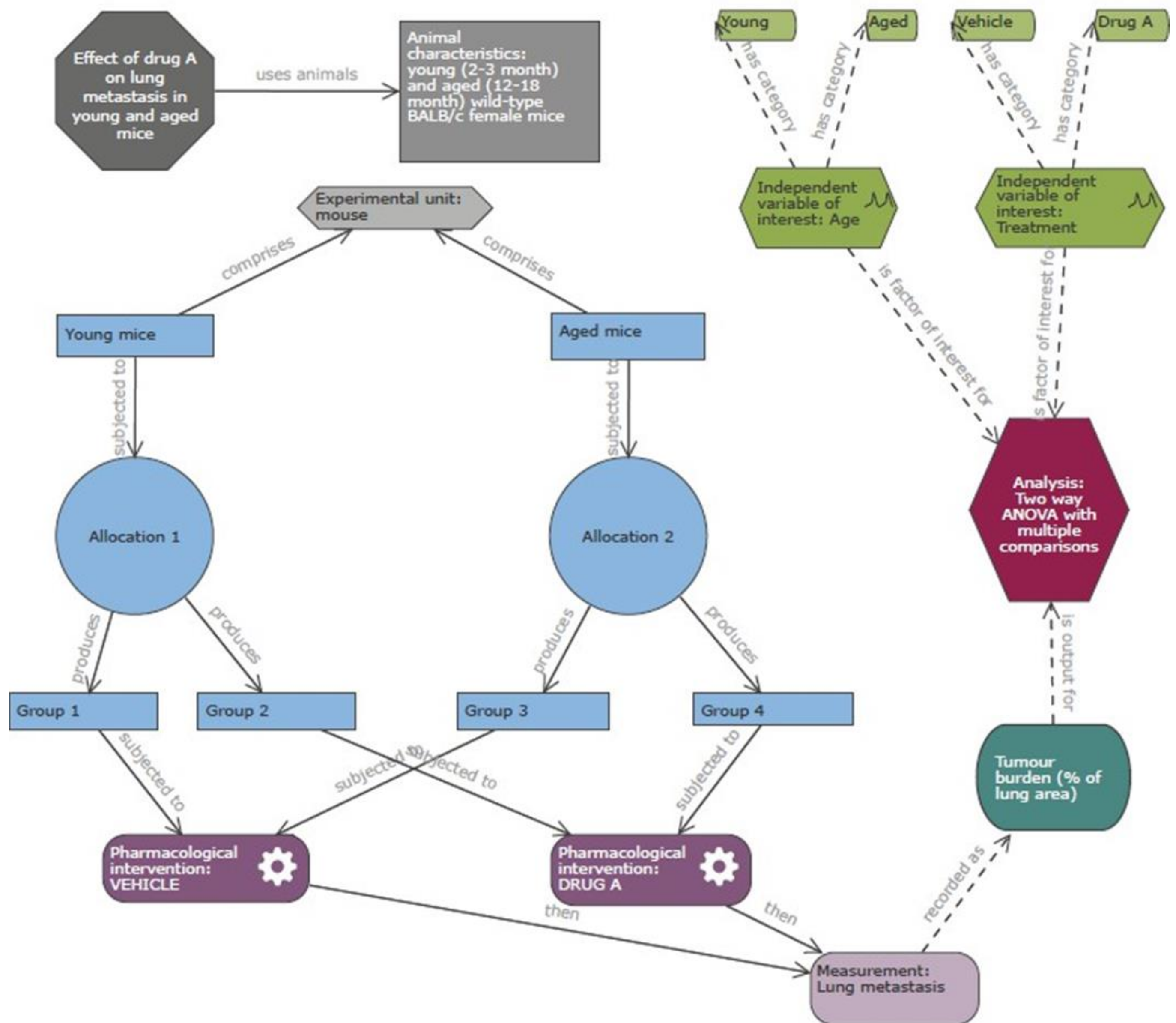
How have you estimated the numbers of animals you will use?

We have estimated the numbers of animals we will use by performing calculations using data generated from previous experiments, information from other research papers and with the advice of a statistician. Depending on the type of experiment, the variability observed between animals under the same experimental conditions differs. These calculations take this variability into account for each experiment type to provide an estimate for the minimum number of animals per group we need to ensure experimental findings are robust and significance can be accurately determined.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

I have used the NC3R's Experimental Design Assistant (EDA) online tool to support the design of the experiments (example shown in Figure 1), as well as using the guidance provided on sample size determination and statistical analysis. The EDA diagram is a particularly useful online tool which I will share with PILs working under my Licence to further support them in the planning of their animal experiments.

Figure 1



What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will ensure we obtain the maximum amount of information from each mouse by performing different types of analyses on each tissue sample, and/or collecting multiple tissue samples from each mouse for analysis, meaning fewer animals are required overall. Using highly-sensitive experimental techniques we can maximise the data we get from each tissue sample. Pilot studies will be performed with fewer animals per group where appropriate to determine the optimal experimental timeline, for example with a new tumour model to determine a) the growth rate of the primary tumour and b) the timepoint at which

the tumour develops its own blood supply; or to determine optimal dosing regime for a treatment. We will also share tissue between collaborators and other researchers (for example other researchers working with aged mice in the Institute; for aged mice we will harvest other tissues that can be used by other researchers).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The majority of experiments in this project will involve transplantation of tumour cells in mice, including spontaneous metastasis models (where the primary tumours are grown and the tumour cells spontaneously escape and spread/metastasise), and experimental metastasis models (to model spread/metastasis at specific sites). These methods are simple and efficient ways for investigating cancer growth and metastasis in mice that are well published as being suitably refined to minimise the level of distress and suffering to the animal, and are typically have a short duration compared to other methods of modelling cancer in mice (experiments are typically 2-8 weeks). To study metastasis from primary tumours, tumours need to have reached a certain size to have recruited and activated surrounding, non-cancer cells from the environment and established their own blood supply which enables the tumour cells to escape and spread. Previous experiments with very small tumours have shown no robust reproducibility. Some experiments will involve the induction of a local fibrotic (scarring) response, e.g. in the lungs or the liver, using methods that are well-published and refined to minimise the level of distress and suffering to the animal as much as possible.

Why can't you use animals that are less sentient?

Mice provide us with a physiologically relevant system; they have similar genomes, they enable us to study immune cells, cells in the environment that have important roles in controlling the growth and spread of cancer, and tumour growth, development and spread in mice recapitulates the progression of the disease in humans. Non-mammalian animals are limited because of significant differences in their physiology e.g. immune system. We use adult mice, not embryos or very young animals because they have immature immune systems and have not reached reproductive maturity (important, particularly for studying hormone-dependent cancers such as breast cancer). Moreover, we include older mice (>12 months) in our project because they better reflect the demographics of cancer patients, for example breast cancer patients. With age, a decline in immune function and other changes to the cells in the environment of the tumour cells occurs. Therefore, it is critical to investigate the growth and spread of cancer in an aged setting, to identify changes that occur with ageing that influence these processes but also response to treatment.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice receiving surgery will be given appropriate analgesia (pain relief) to manage pain and post- operative care and receive enhanced monitoring. Mice >15 months will be frequently monitored by staff trained to work with ageing animals and an index will be used to monitor and score their health with humane endpoints are listed in the index (Table 1). For all animal experiments we will consider whether we can end the experiment earlier than the humane endpoint and we expect this to be the case for the majority of animals. However, in some incidences we will need to use mice with significant tumour burden, for example the control cohort in a therapeutic intervention study – ending the experiment too early would not give is sufficient information about the effectiveness of the therapeutic intervention, and therefore would not be an efficient use of these animals. Pilot studies will be performed to determine the growth rate of new tumour cell lines and tumour-bearing mice will be monitored carefully for any clinical signs of cancer at other sites (metastasis).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Relevant published literature will be used as template for experimental design and decision making (Workman et al., 2010. Guidelines for the welfare and use of animals in cancer research. BJC, 102, 1555-1577) as well as NC3R's Experimental Design Assistant (EDA) online tool.

We will follow guidelines of good practice [Morton et al., Lab Animals, 35(1): 1-41 (2001); Workman P, et al. British Journal of Cancer, 102:1555-77 (2010)].

Administration of substances will be undertaken using a combination of volumes, routes and frequencies that themselves will result in no more than transient discomfort and no lasting harm.

Guidelines for Body condition score [Ullman-Cullere, Lab Anim Sci. 1999 Jun;49(3):319-23].

Ageing mice will be monitored and managed according to an index Table 1 which is adapted from Wilkinson et al (2020) Laboratory Animals: 54(3): 225 – 238 doi: 10.1177/0023677219865291.

We will consult the NC3Rs guidelines and monitor refinement where such practices are published (NC3Rs website and elsewhere).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will keep up-to-date with advances in the 3Rs by regularly checking the NC3Rs website and information displayed in the animal unit and from AWERB review meetings. I have also signed up to the NC3Rs newsletter and registered for the NC3Rs oncology network and others working under this Project Licence will be encouraged to do so too.

7. The role of time in learning and memory

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Learning, Memory, Behaviour, Time, Rodents

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the project is to investigate the psychological and neural mechanisms of the role of time in learning and memory as measured by behaviour in rodents.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The primary benefit of the research will be the advancement of knowledge. Understanding how the brain works is fundamental for a wide range of disciplines (e.g. Psychology, Neuroscience, Psychiatry, Artificial Intelligence, Ethology). The project will lead to the advancement of psychologically plausible models of brain function.

The work is also important for the investigation of abnormal psychological function in neurological and psychiatric diseases. The work aims to establish the psychological processes that underlie learning and memory and then aims to discover the brain regions and the molecular processes involved.

Identification of the psychological and neural mechanisms of cognition allow the causes and treatments of neuropsychiatric disorders to be determined.

What outputs do you think you will see at the end of this project?

The project will lead to publications in high impact, peer-reviewed psychology and behavioural neuroscience journals. The research will be presented at national and international conferences.

Who or what will benefit from these outputs, and how?

The project will provide information that will advance our knowledge of how learning is achieved in the brain. This is of fundamental importance for a wide range of academic disciplines such as Psychology, Neuroscience, Psychiatry, Artificial Intelligence, Ethology. In addition, identifying the psychological processes and neural substrates responsible for normal cognition will aid our understanding of abnormal cognitive processes that occur in neuropsychiatric disorders. Therefore, the work will benefit the research group and researchers at their university interested in the neural basis of cognition and other laboratories across the world as well as pharmaceutical companies.

How will you look to maximise the outputs of this work?

Work will be published in open access journals or will be made open access through the licence holder's university's open access repository. Where possible, data and materials will be made available online or will be available upon request. The findings will be presented at conferences.

Species and numbers of animals expected to be used

- Mice: 1500
- Rats: 250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

In order to establish the neural substrates that are necessary for learning and memory it is necessary to manipulate neural function in a manner that is not ethical nor practical in humans. Computational models, whilst useful for generating novel predictions, rely on empirical data from experiments.

Although I hope that the work will lead to the development of computational models that will determine future research directions, they will not, ultimately, replace the need for the animal research proposed. Therefore, the work in mice and rats is necessary for understanding the neurobiology of learning and memory.

Breeding of genetically modified mice requires the use of mice through all stages of life. However, animals will be tested in adulthood (approximately from 10 weeks old in mice and 12 weeks old in rats).

Typically, what will be done to an animal used in your project?

Animals will undergo behavioural testing for the assessment of learning and memory. This may involve undergoing a number of behaviour tests in order to characterise the behavioural phenotype and to isolate the psychological processes involved by dissociating effects across behavioural procedures.

Behaviour may be motivated by food or water restriction or by fear (e.g., foot shock).

In order to identify the brain regions and mechanisms involved in learning, animals may receive systemic drug administration and/or intracranial surgery for the purpose of brain lesions, drug infusion and/or intracerebral gene transfer.

Animals will typically receive behaviour testing for 3-5 months.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of the work will involve behavioural studies in which the level of severity is mild. For some studies animals will receive drugs by systemic injections or will undergo surgical procedures so that substances can be administered directly into the brain. These procedures will be of a moderate level of severity. The effects of these moderate procedures will be specific to cognition and behavioural performance on learning and memory tasks. However, animals will require a period of time to recover from surgery before returning to a level of health at which the behavioural work can be conducted.

Animals will be killed humanely at the end of the study. In some circumstances it will be necessary to collect brain tissue for analysis under terminal anaesthesia.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Animals that only undergo behavioural testing in the absence of any invasive neural manipulation will only receive a mild severity. Approximately 60-70% of animals of mice and rats will receive a maximum severity of mild.

Animals that receive intracranial surgery will receive a moderate severity. 100% of animals used in protocol 6 will be classed as moderate.

What will happen to animals used in this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In order to establish the neural substrates that are necessary for learning and memory it is necessary to manipulate neural function in a manner that is not ethical nor practical in humans.

Which non-animal alternatives did you consider for use in this project?

Computational models designed to account various aspects of the existing literature on the neural basis of learning and memory.

Why were they not suitable?

Computational models, whilst useful for generating novel predictions, rely on empirical data from experiments. Therefore, although I hope that the work will lead to the development of computational models that will determine future research directions, they will not, ultimately, replace the need for the animal research proposed.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers are based on the number of animals that can be feasibly bred and tested over a five year period and that are necessary for addressing the scientific aims of the project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The number of animals used will be kept to the minimum necessary to achieve the scientific goals by several means. First, where appropriate, with behavioural studies, manipulations of different factors will be conducted within the same animal. This will reduce the total number of animals necessary. Second, counterbalancing of non-crucial factors will rule out potential non-specific explanations of the results.

This will reduce the total number of experiments necessary to reach conclusions. Third, statistical analyses have been conducted to calculate the numbers of animals necessary to avoid false negatives. Fourth, procedures will be constantly evaluated with the aim of increasing sensitivity of manipulations and measures. This will ultimately lead to decreasing the numbers of animals necessary for answering specific questions by increasing the effect size of manipulations.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

By testing on a number of behavioural procedures it is possible to derive a rich and precise characterisation of psychological function within individual animals.

Tissue at the end of the procedure will be shared where possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Rodents will be used. For the behavioural tests, we have developed procedures that allow assessment of learning and memory with the least amount of stress. These include appetitively motivated procedures that take advantage of rodents natural foraging behaviour. Aversive motivation is used only when it is necessary for studying the type of learning (e.g., fear) or for assessing the rate of learning that is necessary for studying a particular psychological process.

Why can't you use animals that are less sentient?

Rodents will be used because (i) there are clear structural and functional equivalents between rodent brains and human brains and common brain structures for temporal processing have been found in mice and humans. (ii) Cognitive states can be studied easily in rodents and they are the lowest vertebrate group in which the specific behavioural tasks required to analyse the role of time in learning in memory have been developed. (iii) Genetically altered rodents provide a means of examining the functions of specific genes, physiological processes, and anatomical systems in cognition.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For behavioural procedures, all experimenters are extensively trained in animal handling and the requirements of the conducting the behavioural task. This reduces potential stress caused to animals. The amount of appetitive and aversive motivation required for behavioural tasks is closely monitored such that the minimum required is used.

For surgical procedures, animals are closely monitored during and after procedures and are given post-operative pain relief until fully recovered.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All work is conducted in line with the following guidelines:

- Code of Practice for Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes

- LASA Guidelines
- RSPCA Animals in Science guidelines
- UFAW Guidelines and Publications
- NC3R's and Procedures with Care
- ARRIVE and PREPARE guidelines

For advances that are specifically relevant to the surgical and behavioural procedures used, we will regularly review the literature.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed about 3Rs advances by reading of the relevant literature, attendance at conferences and engaging the material provided on the NC3Rs website and at NC3Rs seminars/events. Additionally, the local AWERB, NIO, NACWO, NTCO and NVS regularly inform, and disseminate information regarding reduction, replacement and refinement, including new publications of guidelines and research articles, and presentations and reports from collaborators, peers, and animal welfare bodies. We will regularly review procedures and incorporate advances where appropriate.

8. Treatment and Prevention of Diabetes

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Diabetes, Obesity, Autoimmune, Hyperglycaemia, Glucose

Animal types	Life stages
Mice	adult, juvenile
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This programme of work aims to support the development of new therapies to treat type I and type II diabetes as well as other indications associated with diabetes, such as nerve pain and kidney failure. A new treatment must be effective with minimal, if any, side effects. These lead candidates will be assessed for their effectiveness against untreated disease, placebo controls and a drug known to have an effect on disease development.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Diabetes is a disease that occurs either when the body does not produce enough insulin or when the body cannot effectively use the insulin it produces. Insulin is a hormone that regulates blood glucose. Hyperglycaemia, also called raised blood glucose or raised blood sugar, is a common effect of uncontrolled diabetes that over time leads to serious damage to many of the body's organs, especially the nerves and blood vessels.

In 2014, 8.5% of adults aged 18 years and older had diabetes. In 2019, diabetes was the direct cause of 1.5 million deaths, and 48% of all deaths due to diabetes occurred before the age of 70 years.

Another 460 000 kidney disease deaths were caused by diabetes, and raised blood glucose causes around 20% of cardiovascular deaths. Between 2000 and 2019, there was a 3% increase in age- standardised mortality rates from diabetes. Incidence continues to increase while current treatments only address the symptoms of diabetes, mainly hyperglycaemia, but they are not disease-modifying. They also require lifestyle changes to modify the disease or surgical intervention.

What outputs do you think you will see at the end of this project?

The data generated under this project will provide information on lead compounds' efficacy:

(1) New information on the beneficial effect when compared to that of a placebo (vehicle)

(2) New information on the beneficial effect when compared to that of a reference drug i.e., a drug known to reduce disease manifestations.

All data generated will be analysed prior to being recorded in our archives (electronic files and hard copies). All data from a given study will be compiled into a report comprising: (i) a short Introduction including the aim(s) of the study, (ii) a materials and methods section including an outline of the experimental protocol, a summary of the experimental groups and a description of each readout, (iii) a results section including graphical representations of the analysed data, statistical analyses and interpretations, (iv) a conclusion section with a summary of all findings and (v) a raw data section. The study outcome, whether judged 'positive' (e.g., detection of a beneficial effect of the lead compound when compared to that of a known positive control drug) or 'negative' (e.g., absence of a beneficial effect of the lead compound when compared to that of a vehicle and/or no greater beneficial effect of the lead compound when compared to that of a known positive control drug and/or observed adverse events) will be reported.

The data generated will typically be sufficient for the study sponsor to decide the lead compound's future: (i) assessment of toxicology, prior to being tested in the clinic, the next step in the drug discovery process leading to a novel therapy for diabetes, (ii) additional studies for further assessment of effectiveness of the test compound and its activity or (iii) rejection owing to the lack of safety or effectiveness. Testing the efficacy of novel treatments for type 1 & 2 diabetes will provide data to promote the development of safer, more effective treatments of these diseases.

Initial assessment in the animal may help to determine how well tolerated the compound is, whether any clinical signs are observed following administration. In addition, whether the compound is stable following administration (i.e., does not become transformed or broken up into inactive products in the body) and the duration and location within the body in order to determine the best route and frequency of administration to optimise effectiveness.

Our expertise allows study sponsors to obtain advice on the most suitable model to test their lead compound. The interactions between us and study sponsors at the study design stage and during the studies will allow preventing, anticipating, or rapidly reacting to potential adverse events.

Some data may be used to generate scientific publications. These data will be made available to the scientific community (i) during scientific conferences attended by us or our clients, (ii) upon request through our website, or (iii) through targeted email campaigns and social media.

Some data may be used for statistical analysis in order to refine experimental designs and experimental methods and to reduce the number of animals used in future studies.

Who or what will benefit from these outputs, and how?

Work carried out under this license will test lead candidates for several important characteristics necessary for successful drug development. Lead candidates will be evaluated for their safety, i.e., it will be examined whether introduction of these candidates into the body is not harmful and does not perturb critical body functions. Further, it may be determined whether lead candidates readily enter bloodstream and organs affected by the disease and remain there for sufficient period of time to exert beneficial action. Finally, lead candidates will be assessed for their ability to produce the desired beneficial effect of sufficient strength, e.g., leading to the alleviation of diabetes manifestations. These properties of lead candidates will be compared to those of known treatments (i.e., a clinically relevant drug which has been granted authorisation by the Medicines and Healthcare Products Regulatory

Agency). By contributing to the development of new treatments for diabetes, our project will benefit patients worldwide, providing targeted, effective treatment or therapy reducing the impact of the disease on people's long term health.

How will you look to maximise the outputs of this work?

The outputs of this work will help guide key next steps of the drug development process. Where possible, data will be shared at conferences, social media and in publications. The data will aid future studies, add to our experience to advise clients, and provide information to other scientists.

Species and numbers of animals expected to be used

- Mice: 3000
- Rats: 750

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Many different mouse and rat strains have been established to model various human diseases. The inflammatory and immune responses in these animals can be comparable to those in humans, allowing us to study complex diseases such as diabetes. For example, non-obese diabetic (NOD) mice have an immune system that attacks its own insulin-producing cells, causing high blood glucose levels, as seen in patients with type 1 diabetes, making it a useful model to test drugs for the prevention and treatment of this disease.

The NOD mouse allows modelling of most clinical features of type I diabetes observed in humans and can be used to test whether lead candidates prevent or cure weight loss, high blood sugar, inflammation of the pancreas, low blood insulin levels, and presence of antibodies that mediate an unwanted attack of the body's immune system on its own pancreatic cells producing insulin.

In the NOD mice, diabetes arises spontaneously and gradually over variable periods of time. For a more synchronous and rapid induction of disease manifestations, we may treat inbred or genetically altered mice and rats with streptozotocin (STZ), a chemical compound that destroys insulin-producing cells in the pancreas. Diabetes in this rodent model is accompanied by the damage to the peripheral nerves and kidneys, which is also an important consequence of diabetes in humans.

Additionally, owing to their size, ease of reproduction and handling, many aspects of the immune response, leading to disease caused by inflammation and autoimmunity, have been well characterised in these and other similar rodent strains. Their genetic characteristics are also well established, which aids our understanding of disease development and potential interventions. Thus, mice and rats are the most appropriate species for early assessment of potential novel therapies for many diseases.

Typically, what will be done to an animal used in your project?

Animals used in this project will typically develop aspects of diabetes either spontaneously, owing to their genetic characteristics, or after administration of a disease-inducing chemical substance. The animals' blood glucose levels will be monitored regularly to track disease progression and prevent the animals from becoming excessively diabetic.

Treatments will be administered either before disease onset or after the development of diabetes by routes that may cause some discomfort, requiring the use of general anaesthesia with recovery. In some cases, the chemical may cause some adverse effects including body weight loss. Animals will be monitored, and additional nutrients will be supplied in the form of gel or mashed food on the cage floor as required.

Animals may also be bled in-life for the assessment of blood glucose levels and immune response. This procedure will involve restraint for a short time, so animals will be acclimatised to restrainers in advance to minimise the potential stress involve d. Habituation for blood sampling in unrestrained animals using e.g., vet bed, will be implemented wherever possible. Animals will be killed humanely at the end of the study or

following terminal anaesthesia to allow for perfusion and subsequent treatment of organs in a fixative for histological evaluations or for collection of terminal blood.

Duration of studies will vary. For spontaneous diabetes in NOD mice, a typical duration is 38 weeks, with the maximum duration being determined by the onset of hyperglycaemia. For animals treated with STZ, typical studies will be at least 4 weeks long to allow for clinical signs of diabetic neuropathy to be observed when necessary, but never longer that 8 weeks.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals will be expected to develop diabetes that results in high blood glucose levels. This is associated with increased thirst and urination. Prolonged high blood glucose levels can also damage neurons, which will result in the animal experiencing pain. Unless drugs are being tested for the prevention/treatment of diabetes-induced pain, animals will be terminated as soon as they show signs of pain.

Different potential anti-diabetic therapies will be administered by a range of routes that can some discomfort. Animal behaviour following administration of test articles will be monitored for changes in bodyweight, body condition, coat appearance, gait, breathing and activity. To prevent suffering, animals will be terminated before pain and inflammation exceed a moderate severity level , e.g., abnormal movements, reduced activity levels, body weight loss of 15%, and abnormal breathing.

Moderate signs will not be tolerated for more than 24 hours, and animals will be terminated immediately in the event that severe signs are observed e.g., unresponsiveness to external stimulation, whole-body tremor or laboured breathing.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We expect all animals treated with STZ to experience moderate severity, 90% of NOD animals to experience moderate severity and 10% of NOD animals that do not develop diabetes to experience mild severity.

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Diabetes is a complex disease in which blood glucose regulation is disrupted. Its pathological manifestations involve several organs and systems, including the pancreas,

liver, fat, muscle, brain and immune system. These all interact with each-other in a way that is impossible to be recreated in vitro. Therefore, there is no alternative to the use of a living animal that would allow the objectives to be met and progress potential therapeutics to the clinic.

Which non-animal alternatives did you consider for use in this project?

In vitro assays, cell lines, analysis of blood from human volunteers and human tissue.

Why were they not suitable?

Alternatives to animal models are a great addition in the assessment of test compounds and can help to confirm their mode of action. Most test compounds will have been assessed in vitro by the sponsor or by our team prior to proceeding in vivo. However, due to the additional complexity of the human body and interactions of immune cells, different body systems and pathways, it is not possible to replicate this in vitro, and thus in vivo studies are unfortunately still required to fully understand the mechanism of action and show efficacy of test compounds.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Animal numbers are estimated based on our previous annual Returns of Procedures related to diabetes modelling.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will use of in vitro assays, including potential 2- and 3-dimensional set-ups, to minimise the number of animals required for initial assessment of potential novel compounds. In addition, we will utilise the National Centre for Implementation of Replacement, Reduction and Refinement of Animal Use in Research (NC3R's) guidelines and the experimental design assistant to balance group sizes. Numbers of naïve, untreated or vehicle-treated animals will be kept to a minimum while still allowing meaningful data to be assessed for statistical significance of the effects observed. Objective of the study, the desired effect size, as well as the incidence of disease and variability of the model will impact group sizes. Assessment of previous data and/or the literature will also help guide the group sizes as well as the number of control groups required.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Experimental evidence in published literature, our prior experience, information from breeding establishments about the expected incidence of disease and variability of the

model will help ensure that group numbers and sizes are appropriate and that adequate control groups are included.

Additionally, small pilot studies will allow us to determine optimal dosing strategies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Where possible, we will use genetic models of diabetes that overall remain in good condition, despite high blood sugar level, for example NOD mice. Appropriate models will be assessed by reviewing the literature to ensure the most up-to-date, refined model is used.

The most common experimental procedure that will be used is the measurement of blood glucose levels. We will utilise sensitive blood glucose meters that enable precise glucose level measurement using a very small blood sample from the animal's tail. This will cause only a mild discomfort that lasts a matter of seconds.

Why can’t you use animals that are less sentient?

Blood glucose metabolism is well conserved throughout the animal kingdom, but it is within mammals that it closely maps on to human blood glucose metabolism. Most of our studies will be done in mice. Rats will be occasionally used when the disease aspects cannot be modelled in mice, if the test compound does not work appropriately in mice, or if a larger animal is needed for sampling requirements.

Many of our experiments will require collection of multiple blood samples over time, so terminally anaesthetised animals are not appropriate. In addition, blood glucose metabolism is altered under anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animal suffering will be limited by ensuring that the methods used cause the least amount of harm to the animals. Animals will be monitored frequently for signs of discomfort, and appropriate action will be taken promptly. Animals will be treated or humanely killed if they develop signs of excessive suffering. We will also attempt to reduce, where possible, the number of administrations required, minimise drug administration volumes as well as decrease frequency and volume of in-life samples taken. Animals will be housed in groups and kept in an appropriate environment with sufficient bedding, nesting material and suitable objects that would allow them to express normal behaviour. All staff will be trained in good animal handling procedures. Animals will be always handled gently and humanely, especially those that may be in pain. Additionally, bedding that has increased absorbance

may be used to prevent the accumulation of moisture and urea when the animal's urination increases in frequency.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow the recommended Best Practice Guidelines of Turner et al. 2011 and Diehl 2001 and LASA, as recommended by the Home Office. We will keep up to date by receiving regular updates from the Home Office and LASA, as well as information provided by the IAT, following the literature, internal forums and advice from our dedicated veterinary surgeon.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We keep up to date with NC3Rs guidelines by online updates as well as attending Institute of Animal Technology (IAT) Congress and Laboratory Animal Science Association (LASA) conferences and the Home Office Liaison and Training Information Forum workshops.

We will also regularly monitor the literature to ensure the models we are using are the most appropriate and include additional validation of models where improvement can be made.

9. The role of proteotoxic stress response in disease development

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Proteotoxicity, Stress, Disease, Development, Therapy

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The project aim is to understand how impaired proteotoxic stress response impacts disease development. This stress develops due to the accumulation of damaged / non-functional proteins in the body and causes a variety of health problems, for example, cancer.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This project, eventually, will lead to the understanding of how proteotoxic stress response is involved in disease development. Ultimately, this knowledge will help us to identify potential new therapeutic targets, based on the disease model associated with normal proteotoxic response or impaired response.

What outputs do you think you will see at the end of this project?

A final and successful outcome of the project would be the discovery of new therapeutic targets for the treatment of those diseases that are regulated by a normal or faulty proteotoxic stress response. The results that will be produced via this project will be presented at international conferences or published in high-impact journals. Moreover, the lab will be engaging the public via communication and outreach activities, in science fairs and or by visiting schools.

Who or what will benefit from these outputs, and how?

It has been extensively shown that impaired proteotoxic stress response to misfolded protein and uncontrolled aggregation is the cause of various types of health problems and diseases, including neurodegeneration (disease caused by loss of function of brain cells or loss of brain cells), cancer (diseases derived from the uncontrolled growth and reproduction of cells in a specific area of the body) muscle degeneration, cardiovascular disease (conditions affecting the heart or blood vessels), and autoimmune disorders (condition arising from an abnormal immune response to a functioning body part). Hence, it is crucial to understand the underlying mechanisms of proteotoxic stress response that play a key role in these diseases and to identify potential novel targets for their prevention and treatment. Thus, some questions must be asked, such as: How impaired proteotoxic response alter the development of diseases? How can we effectively target proteotoxic stress to identify new therapeutic targets and develop novel therapeutic strategies?

One of the first disease models that this project will cover is cancer disease, which is strongly associated with proteotoxic stress. Cancer is a modern medical challenge that is very difficult to treat, where in some cases can be in remission, but often can relapse back even more aggressively. It results from the progressive spreading and migration in the body of uncontrolled dividing cells, which ultimately destroy tissues and organs. Cancer is a broad term used to describe a plethora of different and variable types even among the same organs or tissues. For example, there are various types of breast cancer (such as triple-negative breast cancer and invasive breast cancer), gastrointestinal cancers affecting organs in the gastrointestinal tract (including colorectal cancer, intestinal cancer, and gastric cancer), and different types of brain cancers like medulloblastoma, glioblastoma, and neuroblastoma. Lung cancer can also be categorized into subtypes like small cell lung cancer, mesothelioma, and non-small cell lung cancer.

In 2020, worldwide, more than 19.3 million people were diagnosed with cancer, and over 9.9 million died from it. Currently, the number of cancer diagnoses is projected to increase by 47% by 2040. In the UK, it is estimated that over 370 thousand people are diagnosed with cancer each year, almost 1,000 per day, with a higher incidence rate among people over 85. The cost of cancer, as estimated by the NHS, is £1.4 million per individual per year. This comprehends the cost of diagnosis and treatment, without considering the economic and social cost for every individual, making it an economic and social burden.

Cancer is intricately linked to proteotoxic stress, which occurs when cells have too many damaged or misfolded proteins, due to cellular processes involved in both disease progression-development and stress response. This situation can overwhelm the cell's ability to keep proteins in check. In cancer, cells divide rapidly and uncontrollably, increasing the demand for protein production and exacerbating the proteotoxic stress. Mutations in genes can further disrupt the cell's ability to manage proteins. To survive,

cancer cells adapt by producing more heat shock proteins and chaperones, which help manage non-functional proteins via their degradation. These proteins are often found in high levels in tumours, helping cancer cells survive, develop and grow. Cancer cells also have enhanced proteasome activity, a cell machinery that breaks down damaged proteins, to clear out the extra load, highlighting the relationship between cancer and proteotoxic stress. The stress and imbalance caused by these mismanaged proteins can further activate signals that encourage cancer metastasis (a process by which cancer cells spread from the original tumour to other parts of the body, forming new tumours). Understanding how proteotoxic stress affects cancer can lead to new treatments. By targeting the proteotoxic stress response in cancer cells, therapies might be able to kill these cells while leaving healthy ones unharmed.

While numerous diseases are known to be associated with proteotoxic stress, our understanding of this complex phenomenon is still evolving, and many other diseases may have yet to be linked to it. Proteotoxic stress, arising from the accumulation of damaged or misfolded proteins within cells, has been implicated in a wide range of health conditions, including neurodegenerative disorders like Alzheimer's and Parkinson's diseases, as well as cancers, and autoimmune and cardiovascular diseases. However, there are undoubtedly numerous other diseases where the connection to proteotoxic stress remains undiscovered. This underscores the importance of ongoing research efforts to elucidate the role of proteotoxic stress in disease pathology development and identify novel therapeutic strategies.

If we are able to successfully answer each of the research questions, we will comprehend how impaired proteotoxic response alters the development of cancer disease, representing our short-term benefits.

Long-term benefits, in the end, ideally, will lead to the identification of new pathways involved in cancer development. These new findings will be crucial to understanding the underlying mechanisms driving tumour growth and progression. Eventually, this new knowledge will shed light on novel potential drug targets for the disease. The identification of novel therapeutic targets for the treatment of cancer diseases would be of utmost importance for patients since it can lead to the possible development of new drugs that will enhance both lifespan and quality of life. Besides the scientific and societal impact, the economic value of novel and effective protective therapies for diseases would be significant.

How will you look to maximise the outputs of this work?

We will provide open access to our publications, and we will have responsible management of research data in line with the FAIR principles. All information about results, tools, and codes required to validate the obtained conclusions will be completely available through public repositories such as GEO (https://www.ncbi.nlm.nih.gov/gds/), Github (https://github.com) and Mendeley Data (https://data.mendeley.com) in order to ensure the reproducibility and transparency of our findings.

Also, in line with the “early and open sharing” philosophy, the primary research manuscripts generated during the proposed project will be available from bioRxiv pre-print server (http://www.biorxiv.org/) as soon as we submit it to a peer-reviewed journal, which preferentially will be an open peer-reviewed journal, also to increase the transparency and reproducibility of generated outputs. Finally, we will implement a thorough process to

ensure the robustness of the obtained results and conclusions by including all relevant negative results in our publications and performing robust and unbiased statistical analysis. The data will be disseminated by presentations at national and international scientific meetings and seminars. Furthermore, our partnerships with other leading researchers and groups in this field from prestigious institutions will be extremely valuable. These collaborations not only provide us with the expertise and resources needed to navigate and investigate the role of proteostasis in cancer development but also offer a unique opportunity to translate our findings from 'bench to bedside.' By bridging the gap between fundamental research and clinical application, we can directly impact patient care and outcomes.

Species and numbers of animals expected to be used

- Mice: 12400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The mouse will be the main experimental model used to investigate the role of proteotoxic stress response in disease development. Unfortunately, in-vitro studies (methods to study the behaviour of mammalian cells in a controlled environment outside of a living organism) cannot be used to understand how genetic modifications result in normal or abnormal physiological processes (function of a living organism or part of its body). The group developed new mouse lines with mutations in genes that impair the ability of the proteotoxic stress response pathway to deal with proteotoxicity. Additionally, preliminary data shows that one strain that was genetically altered is smaller compared to its counterpart animals that did not receive any alteration (wild type), and these animals have less cognitive degeneration at the age of one year, therefore being healthier. As proteotoxicity and the response to it relate to various types of health problems, we aim to understand how impaired proteotoxic stress pathway alters disease development. Therefore, our goal is to phenotype (describe the set of observable characteristics or traits of an organism) our new genetically altered mice throughout their lifespan, from the embryonic stage to adulthood to the age of 24 months, or until they exhibit clinical disease symptoms that may compromise their welfare beyond the limits permitted by this license.

Typically, what will be done to an animal used in your project?

This Project License (PPL) aims to study disease development in the new genetically altered (GA) mice and compare them to wild-type in a group of the same age and sex (cohorts). At first, we will focus on cancer disease development via two models: a chemical-stress model and a genetic model that will be crossed with our mice (typically refers to a breeding process in which two different strains or lines of mice are mated to produce offspring with specific genetic traits or characteristics).

Initially, the animals can go through embryo transfer, where an egg is fertilised outside of an animal’s body to make an embryo (In-vitro fertilisation - IVF), and a medical procedure that cuts the supply of sperm to semen (vasectomy), to establish the GA mice lines.

Typically, chemical stressors (substances that cause cellular stress, causing errors and issues to their normal function) for tumour induction will be administrated to the mice, using administration routes, for example, intraperitoneal injection (IP – injection into the body cavity), subcutaneous (SC – injection under the skin) injection and or diet. Or the mice will be crossbred with spontaneous tumour- developing GA mice. Tumour development will be monitored using non-invasive imaging such as MRI or PET. MRI is a non-invasive medical imaging system that produces detailed images of the internal structure of the body; PET is a non-invasive medical imaging system that detects cells that act abnormally in the body. These techniques will be very important to determine tumour growth and its size, while the animal is unconscious. The animal before non-invasive imaging must undergo food withdrawal, this will guarantee that nutrients or other substances will not affect negatively the images, and therefore, reduce the need to further distress the animal with a second round of imaging.

Additionally, blood samples will be taken approximately once a month, pre- and post-tumour induction, for biomarkers (A biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease) evaluation. Blood sampling will be conducted according to specific criteria, which include not collecting more than 15% of the total blood volume.

During imaging, mice will be kept under anaesthesia to prevent distress or the need to repeat the procedure due to movement.

Furthermore, animals could be aged up to 24 months. Mice, at the end of the experiment, will be injected with cell labelling agents using standard routes (subcutaneous, intraperitoneal). Thereafter mice will be killed humanely at specific time points and tissues will be collected for RNA/DNA (RNA: coded information to be used by cells to build proteins) and protein analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

During the project mice can undergo embryo transfer or (IVF). Mice that will undergo surgical embryo transfer, will experience transient post-operative pain and discomfort. Animals that go through IVF will experience mild transient discomfort and no lasting harm. In addition, mice might be undergoing vasectomy surgery will experience short-lived post-operative pain and discomfort.

The project will focus initially on cancer disease models. Therefore, when using spontaneously developing tumours GA mice models or via a chemical-stress model, cancer will develop and grow in these animals. These animals might experience distress and perhaps pain towards the end of the study when the tumours are developed. Nonetheless, it is not possible to predict the nature or severity of crossbreeding our new GA mice with spontaneous GA cancer model mice, or via chemical induction. In addition, determining the rate by which our new GA mice will develop tumours is not feasible. We, therefore, will follow the timing for cancer disease development and guidelines that can be found in literature or through our collaborators. Different cancer models have different timing for their development, however, one of the first cancer disease models that we will explore is Colorectal Cancer (CRC). This model of cancer will be developed for GA mice in 20 weeks, on the other hand, the chemical model IP of Azoxymethane (AOM: is a carcinogenic chemical compound used in biological research. It is particularly effective for

the induction of colon cancer) with three cycles of Dextran Sodium Sulphate (DSS: a chemical compound that causes intestinal injuries if ingested) and recovery, should develop tumours in a very similar timely manner. These observations are made with wild-type mice, so the prediction or speculation on when our new GA mice will develop CRC cannot be anticipated.

Once mice have developed cancer they will be treated with known chemotherapeutic agents or novel ones, with the intent to cure and alleviate the tumour burden. Additionally, we will be able to assess if the animal harbouring our mutation will respond differently to the treatment. These agents can be administrated via IP, orally (oral gavage – precise administration of substances directly into the animal stomach), SC or via diet - food or water.

In the pursuit of understanding how proteotoxic stress impacts disease development mice can be kept and maintained for ageing until they reach a maximum of 24 months of age. However, as it will occur naturally, animals that live beyond 12 months may experience conditions associated with the age, such as weight loss, reduced appetite, reduced movement, skin and eye problems, fur changes, malocclusion (teeth are not aligned properly), and in some cases even spontaneous tumour development. Additionally, growth and development abnormalities may be observable for certain strains.

During disease development, a procedure that will help us to determine the progression state of the disease is non-invasive imaging e.g. MRI or PET, therefore, helping to prevent pointless distress or pain to the animals. These techniques require the mice to be under anaesthesia. Mice will have no more than ten anaesthetics throughout their lifetime and only one in a 24-hour period. In case after MRI or PET mice do not fully recover from the anaesthesia within 24 hours (eating, drinking, and returning to normal behaviour) they will be humanely killed. Mice can be imaged for up to 2 hours after up to 4 hours of food withdrawal.

During blood sample collection from superficial vessels (saphenous veins), animals will experience mild and transient discomfort. However, if blood has to be collected from mice that are towards the end stage of disease development, they might be already in distress or pain, there we might implement administration of analgesia as described before.

Animals will experience mild and transient discomfort during intraperitoneal and subcutaneous injections for the administration of disease-inducing agents, chemical stressors, and cell labelling agents.

During our studies, we will make use of agents such as disease-inducing agents, and chemical stressors that could cause adverse effects. Therefore, before starting with the full study we will implement pilot studies to assess any potential adverse effects on the new mutant mice compared to the wild type. During these pilot studies, we will be assessing the dose range and frequencies (as number of injections, days of administration, concentration of the different agents) regime for the different agents in disease development.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice:

1) Moderate 50.3%
2) Mild 49.7%

What will happen to animals used in this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Disease development is a very complex event that is the result of the combination and intertwining of several processes at the molecular, cellular, physiological, microenvironmental and developmental levels. To study it, we sought to get as much closer as possible to the human system, therefore, we chose to use mice as in vivo models to achieve our intent. The reason why is that in-vitro research such as cell culture, organoids (organoids are three-dimensional tissue cultures that are derived from cells), tissue culture, and computational methods are mainly used in parallel and complement the in vivo studies. In addition, in-vitro assays alone cannot adequately model the complete array of events during disease development and the behavioural interactions necessary to fully understand its processes.

Which non-animal alternatives did you consider for use in this project?

To achieve our goal in this project we consider several non-animal alternatives that will help with the reach of our goal, understanding how proteotoxic stress is involved in disease development. In addition, we will be consulting FRAME (Fund for the Replacement of Animals in Medical Experiments - Searching for Alternatives | FRAME) to be updated to date with possible alternatives that will help us during our project.

In parallel to the in vivo work, we will use primary cells and cells from abnormal tissues or control ones to:

- form mouse-derived organoids (MDO). They will allow us to have preliminary information on treatment against the disease model, allowing us to test known compounds or novel ones.

- primary cells, such as Mouse Embryonic Fibroblast (MEFs – cells isolated from mouse embryos). We will use MEFs to study the molecular and cellular consequences of the impaired proteotoxic stress response.

These models will not recapitulate the complexity of an animal disease model, on the other hand, the animals can be spared from pharmacological (drug) treatments, that can be screened in those aforehand mentioned non-animal alternatives.

Why were they not suitable?

Non-animal models are not suitable to study the fullness of diseases because they will not replicate the heterogeneity (the quality or state of being diverse in characteristic or content) and complexity.

Moreover, cells or organoids are cultured in an environment that is very different from the live one. Furthermore, cells in culture experience a variable and abnormal oxygen tension that is not experienced in the animal's tissue. They are usually cultured in high concentrations of glucose, growth factors, survival factors and secreted substances derived from cows. Additionally, organoids are still unable to model complex interactions of multiple cell types, such as cells from immune system, connective tissue, blood, etc. Therefore, organoids and cell culture are not suitable for sole use and will be used in parallel with mice work.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

During this project we will investigate the role of proteotoxic stress in different disease models, for this reason, we will be studying several mouse lines. There will be at least 8 different mouse lines with different mutations in proteotoxic stress pathways over 5 years. Mice, in most of cases needed to be homozygous cohorts (two of the same mutant alleles are inherited).

- The estimation of group size and use per different experiment will be derived from the analysis of published work and preliminary experiments. Moreover, we will be seeking assistance from statisticians and collaborators. Variability between experimental groups will be limited by using closely related mouse strains raised in a controlled environment, free from specific diseases, fed a uniform diet and matched for age and sex.

- Dose levels of disease-inducing agents and pharmaceutical therapies will be defined from pilot studies and preliminary experiments.

- We will maximise the data output from a single animal, collecting as many as possible tissues, that are going to be analysed for protein levels, RNA / DNA sequence, and biomarkers. All the data generated, and analysis are going to be supervised and controlled by statisticians.

- We will implement the use of technicians and experienced users for blinded and randomised imaging and analysis.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will regularly employ websites such as:

- Norecopa (https://norecopa.no/), PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) Guidelines: https://norecopa.no/prepare

- NC3Rs (https://www.nc3rs.org.uk/) and its e-newsletter. We will take advice from dedicated technicians within the animal units, and collaborators that have experience and expertise on their side. In addition, we will consider the NC3Rs' experimental design guidance and experimental design assistant (EDA) to plan our experimental design, practical steps and statistical analysis utilising the advice and support for randomisation and blinding, sample size calculations and appropriate statistical analysis methods.

To reduce the number of animals examined to reach data significance for any experimental assay, we will minimise the phenotypic variability via implementing the use of age and gender matched cohorts, born within a window of approximately seven days. Furthermore, genetic background is well known to influence phenotypic variation. Therefore, to avoid variance associated with segregating modifiers, new mutations or well establish ones are generated and maintained on a defined and uniform genetic background. Before a newly generated mice strains undergo behavioural assessment, analysis will be carried out on the target gene(s) to ensure it is appropriately modified.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

During this project when and where there is a need to use alternative genetic backgrounds for certain tests or assays, we will implement the use of pilot studies. The idea is that these pilots will provide us with baseline data and experiment feasibility, so we will focus our attention on these findings avoiding the inappropriate use of mice. If we will need the creation of a new strain; a small colony will be generated on the breeding protocol of this licence. Initially, we will assess for lethality (deletion or modification of genes in animals may cause embryonic or early postnatal death due to developmental abnormalities) and welfare before larger cohorts go through the developmental/phenotyping steps of this protocol. Wherever possible, we will exploit at fullness the mice, performing multiple experiments and collecting as many tissues as possible from an individual mouse. For example, in addition to the abnormal tissue generated from the disease model, the spleen can be collected and used to isolate T cells for toxicology assays (to understand the harmful effects of the environment), the heart can be used to study changes in cardiomyocytes (heart cells), the bone marrow can be flushed and frozen down to generate macrophages to asses inflammation, blood can collected for biomarkers analysis, etc. In addition, these tissues can be used for DNA/RNA/protein extraction for expression analysis, moreover, organoids can be derived in a tissue-specific manner. Embryos can be harvested from females for establishing embryonic stem (ES) cell lines or mouse embryonic fibroblasts. However, in the event we need to share tissues we will become part of the internal tissue-sharing resource (3Rs enquiry list) to facilitate this communication and maximise the outputs of our research.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Throughout this project, we will use mice as an experimental model since the mouse genome has been well-characterised. This allows us to genetically manipulate the genome and thereby study the role of impaired proteotoxicity stress response, induced by individual gene mutations and its role in disease development. To define the consequences of impaired proteotoxic stress response in mice and its consequence, animals with specific mutations in the proteotoxic stress response pathway will be used.

Then, in order to understand how proteotoxicity alters disease development, we will use either (1) mouse models with gene mutations that cause specific diseases in the mice or (2) by administrating disease-inducing agents and chemical stressors. After, ideally, we aim to identify potential treatment targets and therapies for the disease model that we will be generating, preferably using known or novel drug treatments. Moreover, we will compare the response to these treatments of our new GA mice, that are lacking the proteotoxic stress response compared to a wild-type counterpart, or to the mouse GA disease model.

Additionally, to define the development stage of the disease we will consider non-invasive in vivo imaging, such as MRI or PET, which in a cancer disease context are great techniques to visualize the growth of internal tumours such as Liver Cancer, Colorectal Cancer, Brain tumours and many others. Moreover, these techniques will help us to timely and promptly assess tumour size. Therefore, the animal can be humanely killed before pointless suffering. We will also collect blood to test biomarkers, great tools to assess for example the inflammation stage of inflammatory diseases, the spreading of cancer analysing specific cancer biomarkers. This, allows us to preventively stop the metastasizing process, which might cause extra pain and distress to the animals. Blood collection and dosing will be done using the minimum possible volumes and frequencies, guaranteeing that the discomfort for the animal will be the least as possible. Labelling agents will be administered at the end of the experiments, avoiding the animal suffering.

On the other hand, we will implement monitoring, assessment and care techniques that have been perfected by our collaborators over their years of experience.

Why can’t you use animals that are less sentient?

An extended and detailed knowledge of mouse physiology/genetics has allowed us to compare them fully and precisely to the human physiology/genetics, making mouse models one of the most accurate resources in human disease modelling. We aim to define the consequences of impaired proteotoxic stress response in disease development. Therefore, this cannot be done using lower vertebrates such as zebrafish because they are not as closely related to humans as mouse is. Thus, we will use mouse disease models to replicate human diseases. The mouse fits our interest in investigating the role of proteotoxicity in disease development.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All procedures carried out in this protocol will be refined to a high extent, prioritising the welfare of the animal. We will consult published guidelines to ensure updated refinement of the disease that we will induce in our mice, in addition, we will make use of the IMPROVE guidelines on the NC3Rs website and ARRIVE Guidelines (to maximize the quality and reliability of other research).

To ensure the welfare of the animal, we will guarantee that:

- the animals are regularly monitored by experienced researchers and technicians. This will help to not exceed pre-determined endpoints and decrease the amount of distress caused by inexperienced handling.

- the mice are going to be handled by the same set of technicians. In doing so the animals can get acclimatised to being handled by the same persons and therefore be less stressed during routine procedures. Furthermore, the animal will be handled using less stressful handling techniques such as tunnel handling and cupping.

- animals are going to be housed in groups and according to the best recommendations we will implement the best enrichment and nesting materials. In the case of male fighting, we will separate them to avoid fighting wounds.

- mice undergoing procedures will be monitored weekly. If signs of distress or pain (e.g., weight loss) are observed, the animals will be checked daily. We will implement a scoring system and procedures-health observation cards to ensure technicians are always aware of the mice’s condition and the type of procedures they have undergone.

- study groups that will be administered with disease-inducing agents/ chemical stressors to induce disease are going to be monitored daily for the following 5 days. Once this initial period is over, the mice will be monitored weekly. To prevent signs of ill health we can implement the administration of analgesia via sugary substances (peanut butter, Nutella or other), we can also provide extra warmth using heating pads and provide food on the cage floor. This positive reinforcement can be also implemented in case of surgery pre- and post for the administration of analgesia.

- mice that develop the disease can lose weight, usually in a gradual way. However, when the weight loss is detected, we will provide them with wet foods, such as mash, or gel packs. If the animal, will not recover weight after implementing this measure they will be culled.

- animals undergoing non-invasive imaging will be placed under anaesthesia to minimize distress and prevent movement that could necessitate retaking images, which would further distress the animal.

- blood sampling will be done collecting the minimum possible volume and frequencies, which will guarantee the animal welfare but enough material to produce significant data. Blood sample collection will be via saphenous veins, which will cause the least mild and transient discomfort.

Nevertheless, all procedures, experiments and housing will be continually evaluated, reviewed, and refined to minimise and reduce experimental duration, animal numbers and suffering while maintaining or improving scientific benefits. To implement and facilitate this, we will request regular meetings with the lab and collaborators.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The published best practices that we will be following during this project are:

- LASA guidance for surgery and aseptic techniques https://www.lasa.co.uk/current_publications/.

- guidelines (PREPARE) before initiating any experimental study to aid in the planning of each stage (https://norecopa.no/PREPARE).

- guidelines (ARRIVE) to help in the design, analysis and reporting of all studies (www.arriveguidelines.org).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will routinely consult websites such as:

- Norecopa (https://norecopa.no/)

- NC3Rs (https://www.nc3rs.org.uk/) and read the NC3Rs e-newsletter.

We will take advice from NVS, NACWO, and technicians within the animal units, and we will seek advice and help from our collaborators. Furthermore, we will be questioning NIO for valuable

information regarding the 3Rs, Additional support will be sought in:

- ATLA (Alternatives to Laboratory Animals) Journal: https://journals.sagepub.com/home/atla

- LASA Guidelines: https://www.lasa.co.uk/current_publications/.

- 3Rs enquiry list

10. Production, maintenance, cryopreservation and rederivation of genetically altered (GA) mice, rats and zebrafish and associated wild- types

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Production, Maintenance, Genetically altered, Cryopreservation

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant
Rats	adult, embryo, neonate, juvenile, pregnant
Zebra fish (Danio rerio)	adult, embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this licence is to produce, cryopreserve, breed and maintain genetically altered (GA) lines of mice, rats, and zebrafish of a mild or moderate severity for existing PPLs and associated research programs already in place at the establishment. We will also offer a cryopreservation and rederivation service for existing PPLs and associated research programs already in place at the establishment, this will allow a high and consistent standard of breeding and maintenance to the scientific community. We may also offer our services to researchers at other UK institutions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Most genetically altered animals are used in studies to understand the functions of different genes and to recreate human genetic diseases. Cryopreservation allows users to put their projects on hold, avoid the generation of unnecessary animals through continuous or intermittent breeding, and prevent the risks of genetic drift when animals are inbred over time, preserving the genetic alteration.

This application is for a Production (Service) Licence to complement and provide support to ongoing and future research projects that are carried out under the Establishment Licence. No research will be carried out under this licence. All species held or produced under this licence will be transferred onto an appropriate project licence to allow research to progress, or they will be cryogenically frozen. This licence will support the reduction in live animal transport in favour of using sperm or eggs to import new lines into the establishment or to export to collaborators, this supports animal welfare and protects the high health status of facilities.

What outputs do you think you will see at the end of this project?

This project will support researchers at the establishment and may be used to support researchers at other UK institutions. This project licence will be used to rederive, maintain and breed existing and incoming strains of mice, rats and zebrafish prior to individuals own project licences being granted. It will also allow individuals to transfer their lines on to this licence so we can cryopreserve important lines, protecting research programs and reducing unnecessary tick-over breeding of lines. This project licence will allow us to maintain the high health status of our colonies at the establishment and to remove certain pathogens from an existing animal model. This licence will support the reduction in live animal transport in favour of using sperm or eggs to import new lines into the establishment or to export to collaborators, this supports animal welfare and protects the high health status of facilities.

Cryopreservation and rederivation can be used to support researchers who have sensitive lines that are in danger of being lost due to breeding problems.

Who or what will benefit from these outputs, and how?

This service will allow new researchers joining the establishment to avoid delays associated with the confirmation of the health status of live animals and Project Licence application time. It also allows us to maintain the Specified Pathogen Free (SPF) barrier within the facilities thereby helping to maintain the health status, reduce the risk of pathogens spreading to our existing colonies and reduces variability/increases reproducibility of research. The avoidance of delay and barrier maintenance results in a reduction in the total number of animals used.

The provision of this service supports the 3Rs (replacement, reduction, refinement) as it will allow existing users to transfer lines onto this service PPL prior to cryopreservation. Cryopreservation also allows for lines to be archived for future use rather than maintain a live colony with continuous or intermittent breeding leading to a reduction in animal numbers.

The production process covered under this PPL will also allow the technical team to develop the relevant skills needed to perfect these processes through training and reaching out to other establishments. Having a small team will ensure that a high level of

competency is achieved throughout these procedures and having this skill base will result in a more proficient service.

How will you look to maximise the outputs of this work?

An in-house cryopreservation service is more financially attractive to researchers, and in combination with rederivation this service gives a complete package which gives researchers confidence that their lines are safe and can be recovered.

Species and numbers of animals expected to be used

- Mice: 20250
- Rats: 1500
- Zebra fish (Danio rerio): 7500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

These are the GA animals used routinely at our Establishment. All life stages are required to generate the outcomes of this licence.

Typically, what will be done to an animal used in your project?

During superovulation, typically female mice and rats will experience mild, transient pain and no lasting harm from administration of substances by injection using standard routes (intra peritoneal). Animals will typically undergo superovulation once, but some may be reused under specific circumstances, i.e. limited transgenic females, if approved by the Named Veterinary Surgeon (NVS). Rodents will be euthanised by a schedule 1 method at the appropriate time to harvest oocytes/blastocysts/embryos post mortem.

Female mice and rats used as embryo recipients may undergo surgical embryo transfer in which they will experience short-lived post-operative pain and discomfort, or non-surgical embryo transfer in which they will experience no more than mild transient discomfort and no lasting harm. Animals which undergo surgical embryo transfer will undergo the procedure once. Animals which undergo non- surgical embryo transfer may undergo the procedure up to two times or may undergo non-surgical embryo transfer once and surgical embryo transfer once.

Male mice and rats will typically experience short-lived post operative pain and discomfort following surgery for vasectomy. Animals will undergo the procedure once. The vasectomised animals will be used to induce pseudopregnancy in female mice and rats prior to embryo transfer. These male mice and rats may be singly housed after being used to induce pseudopregnancy in females to prevent aggression or will be co-housed with a companion female where possible.

Following local guidelines, mice and rats used for breeding and maintenance of GA animals will experience natural mating on no more than a number of occasions as per local guidelines. Mice and rats may undergo genotyping which will require the removal of a

small amount of tissue during ear notching, they will experience no more than mild, transient pain. Offspring may be maintained by methods appropriate to their genetic alteration until they reach a maximum age of 12 months.

Male and female fish will experience no more than mild, transient discomfort caused by gentle pressure or stroking of their sides during gamete harvest. Fish may be grown and maintained until they reach a maximum of 18 months of age. Fish may undergo genotyping which will require the removal of a small amount of tissue from the most appropriate site or skin swabbing and such that normal behaviour is not compromised, or the micro-abrasion of embryos. Advice from the NVS will be sought regarding the use of analgesia following fin clipping for genotyping in fish aged over 5 days post fertilisation (dpf).

Fish used for the breeding and maintenance of GA zebrafish will be paired for natural spawning.

What are the expected impacts and/or adverse effects for the animals during your project?

During superovulation, typically female mice and rats will experience no more than mild, short-lived pain and no lasting harm from administration of substances by injection using standard routes.

Rodents will be euthanised by a schedule 1 method at the appropriate time to harvest oocytes/blastocysts/embryos post mortem.

Female mice and rats used as embryo recipients may undergo surgical embryo transfer in which they will experience short-lived post-operative pain and discomfort, or non-surgical embryo transfer in which they will experience no more than mild transient discomfort and no lasting harm.

Male mice and rats will typically experience short-lived post operative pain and discomfort following surgery for vasectomy. The vasectomised animals will be used to induce pseudopregnancy in female mice and rats prior to embryo transfer. These male mice and rats may be singly housed after being used to induce pseudopregnancy in females to prevent aggression.

Mice and rats used for breeding and maintenance of GA animals will experience natural mating on a number of occasions. Offspring may be maintained by methods appropriate to their genetic alteration until they reach a maximum of 12 months.

Male and female fish will experience no more than mild, transient discomfort caused by gentle pressure or stroking of their sides during gamete harvest. Fish may be grown and maintained until they reach a maximum of 18 months of age. Fish may undergo genotyping which will require the removal of a small amount of tissue from the most appropriate site and such that normal behaviour is not compromised. Advice from the NVS will be sought regarding the use of analgesia following fin clipping for genotyping in over 5dpf fish.

Fish used for the breeding and maintenance of GA zebrafish will be paired for natural spawning.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

100% of all animals used for superovulation will experience mild severity.

All female mice and rats used as embryo recipients are expected to experience no more than moderate severity. Up to 75% will undergo surgical embryo transfer, and at least 25% will undergo non-surgical embryo transfer.

100% of vasectomised male mice and rats are expected to experience no more than moderate severity.

100% of mice and rats bred and maintained as GA rodents (mild) will experience no more than mild severity. Some will be sub-threshold.

100% of mice and rats bred and maintained as GA rodents (moderate) will experience no more than moderate severity. Some will be sub-threshold.

Up to 90% of GA zebrafish will experience no more than mild severity, at least 10% will be sub- threshold.

What will happen to animals used in this project?

- Killed
- Used in other projects
- Kept alive at the establishment for non-regulated purposes or possible reuse

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The aim of this project is the generation, preservation and rederivation of genetically altered animal models, there is no direct non-animal alternative.

The requirement for animal models is assessed by the researchers that use this service licence and is reviewed during their own application for project licence authority.

Which non-animal alternatives did you consider for use in this project?

N/A for this project.

Why were they not suitable?

In terms of the 3Rs, this licence cannot replace the use of mice, rats, or zebrafish in research as live animals are required for all procedures, but it can (by methods described

in the protocols) help reduce the numbers of animals used and refine their care and welfare.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

As this PPL is a production licence, numbers used will very much depend upon demand. It is difficult to know accurately how many mice, rats, or zebrafish will be required due to variations between strains. Our aim will be to minimise this with careful colony management and monitoring of fertility (average litter size, birth to wean ratio, number of embryos in the case of donor females).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

N/A

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Project technical details are planned and developed in collaboration with the client/researcher, with expert input from the service manager, so that the experimental aims of the project are highly likely to be achieved. All breeding programs will be reviewed regularly to match production with anticipated demand.

Sterile, vasectomised male animals are tested before being used.

Colonies will be managed by ensuring that lines maintained are kept at the lowest possible size to maintain line integrity. Colonies will be managed and consideration taken in regards to breeding performance of strains and careful line maintenance will be followed to ensure no excess animals are produced. Breeding animals will be mated and retired at the appropriate age for the species and strain. Cryopreservation of lines not currently required by a researcher can also be used to minimise the number of animals kept for line maintenance.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice, rats, and zebrafish will be used during this project. These models are used as this project is to cryopreserve, rederive, produce and maintain live mice, rats, and zebrafish. Some embryo transfer recipient animals will undergo non-surgical embryo transfer, this is a refinement of the embryo transfer method. All animals undergoing a surgical procedure will receive appropriate analgesia. Most zebrafish will be subject to natural mating and therefore sub-threshold.

Why can't you use animals that are less sentient?

Less sentient animals cannot be used as this project is to produce and maintain mice, rats, and zebrafish.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Rodent lines undergoing cryopreservation will not always be superovulated as natural mating and freezing of later stage (more durable) embryos is a more reliable method of cryopreservation. This also allows unsuccessfully mated females to be returned to stock or used for subsequent embryo production and avoids the need to inject them.

Any animal undergoing surgery will have an analgesic regime as discussed with the NVS and appropriate post-op care and monitoring. The highest standards of asepsis will be employed.

Stud or vasectomised males may have female companions to avoid single housing.

NVS advice will be sought and followed regarding the use of analgesia after fin clipping of zebrafish or skin swabbing will be used where possible.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Guidance provided by NC3Rs on anaesthesia and post-op care, and colony management will be consulted. The ASRU Efficient Breeding of Genetically Altered Animals Assessment Tool will also be consulted:
Frameworkhttps://assets.publishing.service.gov.uk/media/5c4b0998ed915d38a2f5e2b5/GAA_Framew ork_Oct_18.pdf

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will attend the establishment Culture of Care day and regularly check information provided by the NIO, NC3Rs, IAT, LASA, ISTT and participating in forums and management networks, e.g. Animal Welfare Discussion Board will be used as resources of information about advances in the 3Rs. In addition, information sharing, and best practice will ensure that any advances relevant to this project of work may be implemented.

11. Improving outcomes in complicated pregnancies

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Pregnancy, Fetal growth restriction, Preeclampsia, Placenta, Therapy

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The first aim of this project is to better understand how key risk factors, such as obesity, high blood pressure and advanced maternal age, lead to pregnancy complications and poor health outcomes for mothers and their offspring. Through increasing our understanding of pregnancy complications and their risk factors, we then aim to test whether interventions designed to improve maternal health and/or placental function (a key determinant of a baby's growth) can lead to better outcomes for mothers and their babies, both within the pregnancy itself as well as into later life.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Whilst most pregnancies are healthy and result in good outcomes for mothers and their babies, pregnancy complications remain relatively common. Pregnancy losses, such as miscarriage (loss of a pregnancy before 24 weeks of pregnancy, affecting 15-25% of pregnancies in the UK) and stillbirth (death of a baby after 24 weeks of pregnancy, affecting around 1 in 250 pregnancies in the UK) are devastating outcomes and have far-reaching effects on both physical and mental health.

It is difficult to predict which pregnancies may end in loss, but we do know that poor growth of the baby, also called fetal growth restriction (FGR), is one key risk factor. Preeclampsia (PE) is another pregnancy complication that can increase the risk of pregnancy loss. PE is a serious condition of high blood pressure in pregnancy that can cause problems with the mother's brain, heart, kidney and liver function. If left untreated, it can be life-threatening for mother and baby.

As well as increasing risks during pregnancy, there are life-long increased risks of poor health for mothers who experience these pregnancy complications, and for their babies. Babies who are born small are more likely to develop diabetes and heart disease in later life. In addition, women who have had preeclampsia are at greater risk of developing health problems in later life, including increased risk of heart disease and dementia.

Despite these very serious outcomes, there are currently no effective treatments to prevent fetal growth restriction, preeclampsia or stillbirth, or their long-term consequences. In part this is because we do not fully understand how these conditions arise. The work we will undertake in this project will increase our understanding of how preeclampsia and fetal growth restriction arise, and test whether rational therapeutic interventions can improve pregnancy outcomes as well as long-term maternal and offspring health.

What outputs do you think you will see at the end of this project?

This project will advance our understanding in several key areas of pregnancy and reproductive health. We will generate new knowledge about why women who are older, or who enter pregnancy with high blood pressure or obesity, are more likely to have poor pregnancy outcomes such as pregnancy loss and poor growth of the baby. We will also study the long-term consequences of these higher-risk pregnancies, particularly the consequences of high maternal blood pressure for maternal health in later life, in order to understand what therapeutic strategies might be most useful to improve life-long health in women.

We will present our findings at national and international conferences and publish our research in peer- reviewed publications.

In addition to enhancing our understanding of pregnancy complications associated with specific maternal conditions, we will also test whether we can intervene using treatments either during pregnancy (to prevent or treat pregnancy complications) or in the postnatal period (to improve maternal health following a complicated pregnancy).

By the end of this project, we aim to have generated sufficient data on at least one therapeutic intervention that we think could be used to improve outcomes in pregnant or postnatal women in the future. This information will inform the design of early-phase clinical trials in pregnant women.

Who or what will benefit from these outputs, and how?

Short-term benefits: The beneficiaries of the new knowledge generated in this project will include other academics working within the field of reproductive biology, as well as the wider scientific community and the public.

In terms of academic benefits, we will communicate our findings to a broad range of individuals. These include clinical academics who manage women with high-risk pregnancies, and who will gain a deeper understanding of the reasons why certain women are more at risk of different pregnancy complications.

Long-term benefits: The information gained from this project will lead to a better understanding of common pregnancy complications that affect many families worldwide. It may also lead to the development of new drugs or therapeutic approaches to improve pregnancy outcomes and long-term health of patients. If our findings suggest that certain classes of drugs or therapies may be useful in the treatment or prevention of pregnancy complications and their longer-term consequences, this will open up the possibility of developing new or improved drugs.

Ultimately, the work undertaken in this project has the potential to improve population health in the future and will provide essential information to enable us to advance promising therapies towards clinical trials in women at risk of pregnancy complications in the future.

How will you look to maximise the outputs of this work?

We will publish our findings in open-access journals and present our research as it progresses in both national and international conferences. We are a highly collaborative research group, and where appropriate we will share animal tissues generated from this project and data resources with other researchers in the field. We will continue to publish negative findings in a timely manner to try and ensure that other researchers working on similar models or programmes of work may avoid wastage of animals or resources.

In addition to these academic outputs, our research group has an active public engagement strategy. Sharing findings generated from this project with the public is important not only to share exciting progress in this field, but also to educate people about why it is sometimes necessary to use animals in healthcare research. In preparing and delivering school and community education and engagement events, we are also raising the profile of women's health more generally, which is key to ensure that this area of research gets the attention and funding required to make a difference to population health in the future.

Species and numbers of animals expected to be used

- Mice: 5050

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project will use mice, and predominantly female mice as our primary research questions focus on understanding and treating pregnancy complications. We have chosen to use mice as our research model as we know that the biological pathways involved with a mother's adaptations to pregnancy, as well as early embryo and placental development and later placental function, are very similar between mice and humans.

In order to study the health of the mother and fetus during pregnancy as well as long-term health of the mothers and their offspring beyond pregnancy, we will use animals across a broad range of life stages; from embryonic stages right through until later life. For studies looking at offspring health (both prenatally and postnally) following treatments during pregnancy, we will use both male and female animals, but by necessity the majority of studies in this project will involve female animals.

Typically, what will be done to an animal used in your project?

The majority of animals in our project will be pregnant female mice. Typically, female animals with a known risk factor for pregnancy complications such as obesity (here resulting from feeding animals a high-fat diet before they become pregnant), or high blood pressure (here, due to a genetic alteration in the animals), will be mated with males to generate pregnant females. We will study how the pregnancy progresses, measuring the blood pressure of the pregnant females and studying blood flow to the placenta and the baby using ultrasound techniques exactly as would be done in a human pregnancy.

One difference however is that we will need to keep animals still whilst we carry out these procedures.

This means that we will need to restrain the mice when we measure their blood pressure, and anaesthetise animals in order to carry out the ultrasound imaging.

As we wish to prevent or treat pregnancy complications or improve long-term health, most animals will also be given a drug or therapy that we think can improve maternal and/or fetal health during the pregnancy or reduce the adverse health outcomes of mothers following a complicated pregnancy.

These drugs or therapies may be a dietary supplement or an existing drug that has shown promise either in our non-animal laboratory studies or in the research literature. When it comes to administering treatments, we always try to administer these either in the food or in the drinking water wherever possible, as this is the least disruptive for the animals.

In pregnant animals, we will measure how well fetuses have grown by collecting tissues towards the end of a pregnancy, and in some animals we will study both maternal and offspring health following the birth of the babies from complicated pregnancies that have been treated with drugs or therapies. In this way, we will be able to understand what the long-term impacts of a pregnancy complication and/or a treatment may be.

Wherever possible, we will maintain mice in groups within their cages and they will be given free access to food and water throughout their lives unless we need to remove food for a short period of time to enable us to measure glucose metabolism.

What are the expected impacts and/or adverse effects for the animals during your project?

The feeding of a high-fat diet to induce obesity in mice, prior to and during pregnancy, can cause animals to develop a greasy coat which can then lead to animals over-grooming, a change from normal behaviour. Similarly, animals housed in a low oxygen environment, to mimic a pregnancy where there is insufficient oxygen delivered to the baby (which can occur at high altitudes and is also thought to be a common mechanism that may occur in obesity, hypertension or in mothers of advanced maternal age), may become more subdued as a result of adapting to lower oxygen levels. The duration of these changes in behaviour, should they happen, are expected to be for the length of the diet or low oxygen exposure; 12-16 weeks or 2 weeks, respectively.

For procedures where we need to restrain or anaesthetise animals in order to measure blood pressure or cardiovascular function/blood flow, animals will experience short-term distress as a result. We do not expect these procedures to induce any lasting harm to any animals.

When sampling blood, we always remove the smallest volume possible for the needs of the experiment, in accordance with NC3Rs best practice. Whilst animals will experience mild and transient discomfort from blood sampling, this is not expected to result in any lasting harm for the animals.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

For the mice used in this project, we expect:

25% of animals will experience moderate severity, 50% will experience mild severity and 25% will experience sub-threshold severity.

What will happen to animals used in this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Pregnancy is a particularly complex condition to study as the mother, the baby and the placenta (the organ that supports the growth of the baby) work together to achieve a healthy pregnancy. It is not currently possible to comprehensively study the physiology of pregnancy without using whole animals, although we do conduct our early phase studies (e.g. development of therapeutics) where possible using human tissues and cells to understand some of the more basic aspects of the biology, as outlined below. It is also not

possible to test the safety and effectiveness of new treatments for pregnancy diseases in isolated cells or tissues, as these approaches cannot tell us how the drugs/therapies might travel in the body, be transported across the placenta or affect the fetus. For these reasons, we do need to use some animals in order to achieve the aims of this project.

Which non-animal alternatives did you consider for use in this project?

Wherever possible, we use cells and human tissues (e.g. placental cell lines, human placental tissue and maternal blood vessels from biopsies taken at Caesarean section) to study aspects of placental development and blood vessel function and to test new potential therapies in the laboratory. Our research group is also starting to work towards developing organ-on-a-chip technologies for placental transport and other studies, however these approaches are still in the development stages. Likewise, our clinical collaborators are currently working towards developing in silico human pregnancy modelling approaches. Whilst it is currently too early to replace animals with these emerging technologies, if these models are proven accurate it is likely to lead to a significant reduction in the number of animals used in the future in this field.

Why were they not suitable?

There are not currently any non-animal alternatives that adequately model the interacting systems of mother, baby and placenta together. As outlined above, there are models under development, but none of these are sufficiently advanced or validated to replace animals at this point in time.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

For the studies described in this project, we have calculated the minimum number of animals required to produce reliable results with effects that are biologically or clinically relevant. We have sought statistical support when calculating the numbers of animals we expect to use in the different studies of this project, using either data from our own experiments where possible, or from the literature, to establish the expected variability of different measurements. Where no previous data are available, we will carry out pilot studies to base future power calculations upon.

The majority of our experiments are designed to compare effects between health and disease (e.g. normal pregnancy versus a pregnancy complicated by PE and/or FGR) and/or to assess whether treatment can improve health outcomes in the animals. We make use of factorial study designs where appropriate, to reduce the total number of animals whilst still generating meaningful data.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In planning for this project, we have attended experimental design training courses and have consulted several times with local statisticians to ensure that we are using statistical methods that maximise efficiency in our study designs.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For all studies investigating a new animal model (e.g. a new animal model of PE or FGR described in the scientific literature) we make use of pilot studies in a small number of animals to ensure that the model exhibits the specific features we wish to model in our hands before proceeding to any treatment studies. This is to ensure that the data from all animals used thereafter is translationally relevant.

When conducting studies to understand the effects of treatments across different groups, we use experimental designs that are as efficient as possible to conduct our studies (e.g. factorial study design).

We ensure that tissues taken from animals used in this project are used across as many projects as possible, and where appropriate try to share tissue between researchers from our own group as well as other groups with similar interests.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are the most appropriate model for use in our pregnancy studies, being mammalian, exhibiting similar pregnancy-induced physiological adaptations and having a placental structure and function similar to that of humans.

In terms of our experimental methods, animals are housed in groups wherever possible. Drugs or therapies are delivered in the least invasive way, via the diet or drinking water wherever possible. Any procedures to study a physiological function in an intact animal are done so non-invasively where possible (e.g. ultrasound imaging) or under non-recovery conditions (e.g. terminal anaesthesia for placental transport studies) to minimise harm to the animals. Where this is not possible, for example glucose tolerance testing, we use microsampling to minimise the volume of blood taken.

Why can’t you use animals that are less sentient?

In order to understand mammalian pregnancy and develop treatments that can ultimately be translated into humans, we need to use a mammalian species, with the mouse being the most appropriate and least sentient species for this work.

We will study effects of pregnancy complications and new treatments on developing fetuses at early gestational ages (i.e. at immature life stages) in some of our studies. However, in order to study the effects of these treatments on the pregnant female and to understand the long-term effects of these treatments, we must study adult animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Any animal undergoing an intervention (e.g. administration of a new treatment to a pregnant mouse) will be monitored by researchers for the duration of the treatment period, with increased monitoring during the initial days to ensure there is no change in food or fluid intake. Likewise, animals exposed to hypoxia will have increased monitoring during the period of incubation.

For blood pressure measurements, animals will be habituated to the restraint tubes before measurements are made to minimise the stress of the procedure and improve the reliability of results. For studies where blood pressure will be measured during pregnancy, animals will be habituated before the animals become pregnant.

If any animals undergo recovery surgery for the purposes of implanting a drug delivery device (minipump), then we will ensure animals are given both pre- and post-operative analgesia and undergo increased monitoring until they have made a full recovery.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Our research studies are planned and conducted in accordance with the PREPARE and ARRIVE guidelines to try and ensure that the research we produce is as reliable and reproducible as possible. We are also committed to publishing both positive and negative findings, and have an established track record of doing so.

Researchers working on this project will engage with continued professional development; all researchers will undertake training in experimental design (e.g. as delivered annually by the animal facility) as part of their training within the research group.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Through our institution we receive monthly newsletters where the latest developments from the NC3Rs are communicated to all researchers. We will make use of the 3R's assessment tools throughout the lifecycle of this project, to ensure that any new advances that we can apply to our research are implemented.

12. Neural mechanisms underlying voluntary motor control

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Motor control, Movement, Brain, Muscles, Coordination

Animal types	Life stages
Mice	neonate, juvenile, adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Our aim is to try and understand how the brain plans, initiates and controls complex body movements. Since the early 1900s scientists have been interested in how the brain controls our muscles in order to perform complex actions such as hitting a tennis ball or playing the piano. Although significant progress has been made, it is still unclear how multiple different brain regions coordinate their activity to ensure online motor control.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Movement is the measurable output of mammalian behaviour. Almost everything we do, every thought we have ultimately results in a movement of some kind, whether navigating to find food or interacting with others in a social environment. In this programme of work, we will build on existing data derived from animal models to develop an in-depth understanding of how neurons in the brain generate patterns of activity that are necessary

for planning, initiating and controlling limb movements. Our aim is to generate a ‘functional blueprint’ of motor-related areas of the brain in health, with a view to uncovering potential entry points for therapeutic intervention aimed at alleviating the debilitating symptoms associated with disorders that affect motor control (e.g. stroke, Parkinson’s disease, Rett’s syndrome).

Direct beneficiaries of our work will be local and international science communities who can access our findings via published peer-reviewed journals and through participation in national and international conferences. Although our work is not directly translatable, we will work with relevant agencies to ensure dissemination to health sector partners and pharmaceutical companies.

What outputs do you think you will see at the end of this project?

The measurable output of any behaviour is movement, whether it’s simply breathing, mapping the environment that you are in in order to navigate through it, or performing a complex object manipulation task to achieve a desired goal. Knowing the principal mechanisms that govern complex motor control in health will undoubtedly identify potential entry points for therapeutic intervention in disorders of the brain, the opportunity to build physiologically accurate network brain models, and to generate new brain machine interfaces (BMIs) and artificial intelligence (AI) that can assist in many aspects of everyday life for a patient that has experienced loss of motor control. The main output from this project will be the generation of information and large-scale datasets that will be disseminated via accessible publications and online portals, respectively.

Who or what will benefit from these outputs, and how?

Our multi-level cellular and systems neuroscience approach will generate an in-depth understanding of motor control in the rodent, which can be used as a platform from which to identify generalised mechanisms of motor control across mammalian species. As such, our findings will influence the way people think about motor control in academia, pharmaceutical industries, healthcare sectors and those interested in developing next-generation BMI (brain-machine interfaces - i.e. using neural activity to drive limb prosthesis) or neural-based AI (artificial intelligence - designing a robot that can move and think like a human) technologies. Brain-Machine-Interfaces (BMIs) suffer from poor "understanding" of how neural signals translate into movement control. Our aim is to use animal models to record and manipulate brain activity to try and identify the 'code' that the brain uses to control movement. This in turn will provide the foundation and information required to design novel BMIs that better represent or replicate human control of limb movement. As such the benefits from our research will be realised across different timescales from short- to medium, and with respect to influencing healthcare, longer- term.

How will you look to maximise the outputs of this work?

We aim to share the results from this work in the following ways:

- communication with members of the scientific community through collaborations and at relevant conferences
- publication of our work in open access research journals
- making completed datasets available to the scientific community

- engaging in public engagement through family groups, patients, charities and social media.

Species and numbers of animals expected to be used

- Mice: 5500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The project will investigate how activity in neurons and neural circuits in the intact brain relate to the execution of voluntary movements, and how dysfunction leads to loss of motor control. To this end, the project will assess brain function in juvenile through to adult mice at timepoints where they are able to learn complex motor behaviours. We use mice as our model of choice for the following reasons:

Mice show a remarkably similar brain structure to higher-order mammals including humans providing an accessible model system with which to interrogate the relationship between brain function and behaviour.

It is not possible to explore cognitive functions and how they relate to the planning, selection and execution of different motor behaviours in simpler models such as flies or worms.

A wealth of genetically modified mouse lines exist that can accelerate progress which are not available in other species. This in conjunction with a vast array of techniques and approaches developed for mice provide unprecedented access to the activity of single neurons or dense neuronal networks during behaviour.

Typically, what will be done to an animal used in your project?

A typical experiment will involve mice being trained (either head restrained or freely moving) to perform motor tasks (i.e. pushing a lever, manipulating a joystick or reaching to grab an object) over the course of a few weeks. We will perform recordings of neural activity once the subject has learned the task or across learning using electrophysiological or imaging techniques. This will allow us to correlate neural activity with different aspects of the behaviour. To explore a causal relationship between neural activity and the underlying behaviour a typical experiment will involve some form of manipulation (e.g. optogenetics - use of light-activated channels to control neuronal excitability , chemogenetics - use of novel engineered drugs and receptor combinations to control neuronal excitability or traditional pharmacology) which are all reversible. To complement recordings of neural activity and videos of behaviour we may record muscle activation using electromyography (EMG) via implanted electrodes or less invasive multi-site EMG cuffs. Most experiments will involve surgical procedures to attach head plates, inject substances, record etc but the number of surgical interventions will be kept to a minimum as will the duration of the subsequent behavioural training. All surgical procedures will be performed under general anaesthesia using intra- and post-operative analgesia, typically

lasting between 1 and 4 hours. At the end of each experiment, animals will be humanely killed, and brain tissue used for other purposes such as anatomical mapping of the underlying neuronal circuits.

In some experiments, mouse models of autism spectrum or neurodevelopmental disorders will be used to explore how gene mutations lead to neuronal circuit and behavioural dysfunction. We will use many of the same approaches detailed above to assay changes in phenotype, cognitive functions and motor control when compared to wild type littermates, with welfare scoring conducted throughout the lifetime of the animal.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals that require invasive surgical procedures will be continually monitored. Pain will be controlled during surgery by general anaesthesia and by administering intra- and post-operative analgesics.

Stress will be minimised by habituating animals to the experimental setup prior to each experiment. To facilitate learning food or water restriction may be used. Adverse effects associated with this type of experiments are expected to be minimal. The application of drugs will be focal and restricted to particular brain regions so there should be minimal behavioural side effects. Deaths resulting from anaesthesia or surgical complications are uncommon (<1%) and will be minimised by correct dosing of anaesthetics, by accurate weighing and maintenance of body temperature during and post-surgery e.g. use of heat pads. To increase the rate of recovery and reduce the risk of hypoxia supplemental oxygen will be provided throughout each surgery. Risk of infection will be minimised by good surgical and aseptic practices. During the vast majority of experiments the severity rating will be mild with <=10% being classed as moderate.

The highest severity rating of this programme of work will be associated with the use of genetically altered mouse lines that display progressive loss of motor control with endpoint respiratory failure. We will however conduct the majority of our experiments either when mice are pre-symptomatic or at a stage where motor symptoms are relatively mild. Strict health and welfare surveillance will be conducted at all times and humane endpoints for euthanasia will be strictly adhered to.

At the end of each protocol, animals will be killed by using approved humane methods and tissues from these animals may be used for post hoc histology.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The severity level will not exceed moderate. This applies to all animals used in the project.

We expect that for 90% of animals the severity rating will be mild or subthreshold since they will be used for breeding, behavioural testing and/or administration of compounds followed by humane killing for tissues. The remaining 10% may experience moderate suffering due to the progressive development of motor dysfunction in mouse models of austism spectrum and neurodevelopmental disorders.

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To understand how brain activity relates to behaviour requires the use of animal models and experimentation. A core aim of this research programme is to generate a deeper insight into how different brain regions coordinate their activity to plan, initiate and control complex movements. To do this requires a raft of molecular genetic tools and mutant mouse lines (cre-driver lines) that can facilitate identification of specific cell types or projection pathways of neurons, which is helpful when recording or manipulating neural activity. Given the similarities in brain areas, gross architecture and connectivity, the rodent brain provides an accessible, tractable model for understanding motor control and is the go-to model for preclinical studies of brain function. In addition, animal models of neurodevelopmental disorders provide an important tool to understand disease mechanisms and potential routes for therapeutic intervention prior to clinical trials.

Which non-animal alternatives did you consider for use in this project?

In silico computer modelling is fast becoming a viable alternative to simulate some aspects of neuronal/ circuit function, but as yet computer models do not fully recapitulate the intricacies of brain function. To complement our animal-based experiments we have developed strong collaborations with computational neuroscientists in the UK and USA to explore development of next-generation in silico models of brain function. This is proving to be an exceptionally fruitful approach where we can generate hypotheses using predicitive models that can then be tested in animals through experimentation. This in time will help to minimise the use of animals as the experimental model and in time will replace some forms of animal experimentation.

Why were they not suitable?

Tissue electrophysiology or cell recordings will not reflect how an intact organism performs learning and memory. The targets identified from cells or tissues cannot capture the full complexity from an intact system with various circuit inputs and outputs. Mathematical modelling cannot replace the animal research that provides a holistic consideration on genetics, environments, neurotransmissions, neuromodulation, and circuitse.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Animal numbers have been estimated based on known breeding patterns of wild type and transgenic mice, known effect sizes for the majority of the outlined experimental procedures and over 20 years experience in designing and conducting programmes of neurophysiological research.

Mice: experiments involving mice require the generation of small breeding colonies of wild type and transgenic mice to ensure sufficient offspring for use in experiments. Cohorts of 3 mice per week will be used for behavioural training and recording, with ~10 projects running concurrently. The number of breeding and experimental mice is estimated to be 5500 mice across the 5-year period.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our cohort sizes reflect the minimum number of animals required (given the known effect size) to provide statistical robustness, thus avoiding ambiguity and the possibility of having to re-run experiments to ensure that a statistically significant result is indeed valid. Our experimental plans incorporate a design phase where we ensure that the correct recording or manipulation approach has been selected (i.e. is it the best approach to directly answer the experimental question), a well thought through analysis plan with knowledge of the likely effect sizes, type of data and within and across subject variability. An important aspect of this is to first consider the statistical analysis that will be undertaken to avoid pseudoreplication and incorrect use of statistical tests. Only by taking these factors into consideration is it possible to design a robust fit-for-purpose experiment.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Pilot studies - we routinely use small cohort pilot experiments to explore the different effect sizes that would be expected in any given experiment or to optimise the stereotactic coordinates for recording or viral-mediated transgene expression. The outcome(s) of each pilot experiment are then used to shape the final experimental design.

Power calculations - based upon know effect sizes from the literature or pilot studies will be used to ensure statistical robustness while minimising the number of animals used.

Computational modelling - we use in silico computational modelling which facilitates the identification of key cell-types, circuits or brain regions to target thus reducing the need for explorative experimentation and overall animal numbers.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use wild type and genetically modified (GM) mouse lines as they provide an invaluable set of tools to interrogate the neural mechanism underpinning motor behaviour. GM mice provide a robust model system with which one can use advanced recording and manipulation techniques to investigate how neurons in the brain communicate with each other, and how distributed brain areas coordinate their activity to control limb movement.

To minimise stress during experiments, mice will be handled extensively before and during behavioural training and we will ensure adequate recovery periods between surgeries. It has been well documented that stress reduces the ability of mice to learn simple and complex behavioural tasks so it is within the experimenters' interest to ensure a 'stress free' environment in which the mice can train.

The majority of experiments in this programme of work will have a moderate severity rating due to the use of invasive surgical procedures, brain manipulations and behavioural training. The experimental cost to each animal will be minimised through the effective use of pain relief, general anaesthesia and post-operative analgesia. Principles for good surgical practice will be followed throughout.

Why can't you use animals that are less sentient?

To understand how brain activity relates to behaviour requires the use of animal models that can be trained to perform motor tasks. This precludes the use of animals at earlier developmental stages, or the use of animals with reduced sentience given that we are exploring cognition, sensorimotor learning and motor control. We have chosen to use mice as they provide a robust model of human brain function, while also having genetic tractability and accessibility in terms of neural recording and manipulation techniques.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will adopt several approaches to help minimise welfare costs:

1) Animals will be housed in groups and all cages will be enriched with tubes, objects, climbing ladders/obstacles and running wheels. This will promote healthy interactions and will provide a high degree of novelty and exercise.

2) All our experimental protocols have been designed with reduced stress in mind as this facilitates task engagement and learning. Mice will spend a considerable period of time time being habituating to both the experimenter and experimental setup. Only when mice are calm and fully habitauted do we begin behavoural training or perform recordings/manipulations.

3) We strive to continuously refine our protocols based on in-house developments and ideas and/or best practice suggestions from the published literature. Protocol refinements will be implemented where and when is necessary.

4) All surgeries will be performed under general anaesthesia using aseptic techniques and pain relief will be administered during recovery to minimise distress. We strive to

ensure rapid recovery after surgery as this minimises stress and any long-term effects on behavioural training/performance.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow all best practice guidance provided by our institution. We will also follow procedures that we and others have published that establish refined methods for the specific experimental questions we aim to address.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our Veterinary Services team provide exceptional support to individual research groups, allowing us to stay informed about and implement advances in the 3Rs. The Named Information Officer (NIO) circulates HO guidance notes and welfare organizations' newsletters through emails. The Named Veterinary Surgeon (NVS) team and full time Named Training and Competency Officer (NTCO) work together with PIs to refine procedures and disseminate best practice ideas.

13. Fish Movements and Behaviour

Project duration

5 years 0 months

Project purpose

- Basic research
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Fish movements, Fish behaviour, Fish telemetry, Otolith micro-chemistry

Animal types	Life stages
All other fish	adult, juvenile, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To improve knowledge and understanding of the movements, migrations, patterns of distribution and behaviour of fished and bycaught marine fish populations (excluding endangered species listed in Annex 1 of CITES), in relation to their environment. The knowledge and understanding will be used to provide advice in support of rational management and conservation to stakeholders, national and international governments and other national and international organisations.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Information on all aspects of marine fish migration and distribution in relation to the environment will permit better advice to UK Government in support of national and international commitments to regional management and conservation of marine populations. The advice will be mediated through the Government, but also on behalf of the Government through participation in national and international committees and

organisations (such as the EU December Fisheries Council, The Scientific, Technical and Economic Committee for Fisheries, The International Commission for the Conservation of Atlantic Tuna, and The International Council for the Exploration of the Seas), as well as to relevant stakeholders (e.g. commercial and recreational fishing industry and NGO representatives). The information will also contribute to the development of improved methods for assessing marine fish stocks and will help provide a fundamental understanding of the movements, behaviour and distribution of fish in relation to their environment. In turn, this will improve our capability to advise on the likely impacts of commercial and recreational fisheries on fish stocks and enable the design of fisheries management and conservation efforts aimed at optimisation of fishing effort and avoidance of depletion of threatened species.

The work will also inform on the impacts of capture and release, whether deliberate or unintentional, by improving knowledge of post release mortality, or long-term discard survival of commercial and recreational fish species. This will improve advice on e.g. survivability exemptions from the Common Fisheries Policy landing obligation, whereby unwanted fish can be returned to the sea following capture, rather than retained and landed. Additionally, for species such as some species of sharks, skates and rays, long-term post-release survival from commercial and recreational fisheries will inform the Government (and other relevant bodies) on alternative management strategies to reduce fishing mortality.

What outputs do you think you will see at the end of this project?

The outputs of the work will include:

1. Data
    - individual-based data on migration routes, residence times and residence locations, preferred habitats and patterns of behaviour in relation to those habitats;
    - individual-level impacts of capture on post-release behaviour and survival
2. Information and knowledge
    - new understanding of the relationship between biological indicators and behaviour;
    - improved understanding of stock and population structure, life history strategies and the individual and population response to environmental drivers
3. Publications
    - use the data and knowledge gained in peer review publications and technical reports to enable knowledge transfer to wider society

Who or what will benefit from these outputs, and how?

The short-term benefits will likely be felt locally through advice and measures applied to specific regions or fisheries, and help to meet national or regional statutory obligations for rational and sustainable fisheries and environmental conservation. In the medium and longer term, depending on the extent to which the new knowledge is species- or region-specific, the benefits could be felt nationally and internationally and help to meet national

and international commitments to fisheries or environmental management. Other benefits from the transfer of knowledge to wider society (other scientific institutions and academics, government and policy makers, civil society) will develop over time as the new insights from the work become integrated with existing knowledge.

How will you look to maximise the outputs of this work?

Experimental work will be developed in collaboration with project partners and with a communications team supporting the dissemination of results and major programme highlights.

Species and numbers of animals expected to be used

- Other fish: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The aim of the work is to advance our understanding of the movements and behaviour of marine fish of commercial, recreational and/ or conservation concern. The work requires direct empirical evidence of behaviour and responses to environmental or biological drivers. In general, the size of the individuals used will mostly be adult life stages because the instruments available for use to collect the necessary data are on the scale of several cm in size and are therefore only suitable for individuals large enough to carry them without adverse effects.

Typically, what will be done to an animal used in your project?

Individuals used in the majority of studies will be captured using modified fishing methods (to reduce adverse effects). Discard survival studies, require that standard fishing methods are replicated from commercial or recreational fisheries. Once caught, fish will be held at the side of the vessel, or in tanks for a short period of time prior to the procedure being undertaken. A suitably sized electronic device will be attached externally or implanted internally using appropriate attachment material or surgical techniques. Individuals may be tagged in the water or on the deck if they are very large, but most will be removed from the water temporarily to facilitate handling. The procedure may require anaesthesia and/or analgesia if this reduces the adverse effects during the procedure or afterwards. Small tissue samples, such as blood, muscle, mucus or fin biopsies, may be taken. In some cases, tagging will not be conducted, but tissue samples may be taken. Where feasible, tissue samples collected in this project may be used to help inform stock identification or to assess physiological or biological status.

Individuals will be given a recovery period after the procedure to assess their fitness for release to the wild, at which point they will be discharged from the Act and returned to the sea.

What are the expected impacts and/or adverse effects for the animals during your project?

The procedures are assessed as Mild or Moderate severity. Possible adverse effects include:

1. Pain or distress during the tagging or sampling procedure. If necessary and beneficial, anaesthesia and/or analgesia will be used to minimise this risk. Whether anaesthesia and/or analgesia is used or not, fish will be handled carefully to minimise handling stress and their response to it, and will be placed onto surfaces that will minimise accidental movement. Careful monitoring of the fish will be undertaken throughout the procedure, and until the fish is fully recovered.

2. Infection of tagging wounds or skin (as a result of handling or capture damage). Risk of infection will be minimised by conducting appropriate antiseptic and disinfection procedures and techniques to create aseptic conditions for tagging. Where appropriate, analgesia will be applied to the tagging site to reduce likely pain.

3. Loss of weight or condition due to handling or tissue sampling. This risk is considered small because handling and sampling protocols have been carefully designed on the basis of previous practice and experience. Tissue samples would be scaled appropriately to the size of the individual, and taken from areas of the body where the risk of damage, pain or post-procedural deterioration was minimised.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Expected severities are 76% Mild and 24% Moderate.

What will happen to animals used in this project?

- Killed
- Set free

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The aim of the work is to use electronic telemetry methods (acoustic tags, data storage tags, or pop-up satellite archival tags) together with (where appropriate) otolith microchemistry (stable isotope research) and biological sampling (including genetic analyses and physiological assessment), to advance our understanding of the movements and behaviour of marine fish of commercial, recreational and / or conservation concern (excluding endangered species listed in Annex 1 of CITES). The knowledge being pursued does not exist and is typically species-specific. For this type of investigation there is no appropriate alternative to the use of conscious wild fish (i.e. there is not a non-protected animal alternative).

Which non-animal alternatives did you consider for use in this project?

Procedures will only be undertaken for species and areas where there are gaps in knowledge that cannot be filled with non-animal alternatives. For example, where there is information available on the movement, behaviour or discard survival for the species and area of interest - this information will be used first of all. For example, a non-animal alternative that could be considered to help fill data gaps includes passive acoustics, which can help to quantify seasonal changes in the abundance and biomass of marine species.

Why were they not suitable?

Passive acoustics can be used to understand hotspots for marine species abundance, but it is very difficult or impossible to identify single individuals to species level. Even so, for migratory species, this only provides a discreet period of monitoring in areas where there is acoustics with validation tows. To monitor the behaviour of mobile marine species, at present there is no appropriate alternative to the use of telemetry on conscious wild fish to achieve the aims and objectives.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The experimental methods and numbers of animals used are based on previous experience and research and will be assessed prior to any project being implemented (including support from Cefas statisticians).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All experimental methods and numbers of animals used will be based on published literature and previous experience and research by the Project Licence holder and colleagues at Cefas (including the use of statistical power analysis to assess appropriate numbers for the study aims). As part of Cefas' Animal Welfare and Ethical Review Process, each programme of study is considered by senior staff from our in-house scientific and statistical teams and their sign-off is required before any study is undertaken. The use of electronic tags, rather than simple ID tags, enables the collection of high-quality data on individuals at liberty for up to several years, which provides far greater insight than the information gained from the recapture of ID-tagged fish alone. Depending on the aims of the study (and on individual variability), this can help to reduce the number of individuals required in the study.

Where possible, the use of methods that enable remote transmission of data will be used so that the recovery of data is not dependent upon recapture of the fish and the data recovery: animal use ratio is maximised.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Studies will be undertaken using as few individuals as possible for the required aims. Information collected in previous studies (including where appropriate, from other species), will be taken into account when designing experiments, to ensure that animal numbers are optimised. Recent studies have included the use of statistical power analysis to identify appropriate numbers to include in the study. Data collected from these studies (including tissue samples and electronic tag data) will be used in partnership with other institutes and organisations to help optimise animal use to achieve study aims.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

A range of species including sea bass (Dicentrarchus labrax), spurdog (Squalus acanthias), common blue skate (Dipturus batis), common flapper skate (Dipturus intermedius), porbeagle shark (Lamnanasus), Atlantic bluefin tuna (Thunnus thynnus) etc. need to be studied to achieve the aims and objectives of the project. The methods chosen are based on previous experience and research that has been shown to provide evidence that is valuable in formulating advice on factors that may affect fish populations and possible mitigation.

Why can't you use animals that are less sentient?

The aim of the work is to advance our understanding of the movements and behaviour of marine fish of commercial, recreational and/ or conservation concern in relation to their environment. The study is therefore dependent on collecting species-specific information.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Where appropriate, anaesthesia and/or analgesia will be administered to provide pain relief. All animals will have a post-procedural assessment to ensure their fitness for release to the wild.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Best practice guidelines for fish tagging and sampling were documented at a Workshop on Mark Identification Tagging (WKTAG) in January 2024 and will be used as a basis for developing effective methods. Tag attachment/implantation methods will be continually updated, reviewed and evidence- based alongside tag technology developments to ensure that they are humane and that they minimise the effects on the fish's behaviour, long-term welfare and survival. Published studies on refinement of techniques will be used to help refine protocols and methods. PILh and the PPLh will contribute to working groups and studies to help inform others of best practice that arises from this study.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

With tag technology continually improving, electronic tags become smaller, with increasingly benign attachment/implantation approaches. Tag attachment/implantation methods will be continually updated, reviewed and evidence based alongside tag technology developments to ensure that they are humane and that they minimise the effects on the fish’s behaviour, long-term welfare and survival.

14. Role of microglia and blood-brain barrier in dementia

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Microglia, Blood-brain barrier, Alzheimer's disease, Vascular dementia

Animal types	Life stages
Mice	juvenile, adult, pregnant, neonate, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the project's objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aims of this study include investigating the roles of microglia and blood-brain barrier dysfunction in age- and metabolic disease-related dementia by generating dementia mouse models that closely resemble human dementia conditions; and performing preclinical drug tests in these dementia models.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Dementia is not a natural part of ageing. The patient has an impaired ability to remember, think, or make decisions that interfere with everyday activities.

Alzheimer's disease (AD) and vascular dementia (VaD) are the two most common forms of dementia. Though these conditions affect millions of people worldwide, there is currently no cure or prevention. AD and VaD are complex and progressive diseases in which they are not separated but overlap in nearly 50% of the cases.

Researchers have discovered that abnormal immune responses and blood vessel injury are possibly common conditions in AD and VaD. To develop effective treatments, we need to understand the intricate mechanisms underlying the disease. Understanding the importance of immune cells and the damage of the blood-brain barrier in dementia is crucial.

Immune cells, in particular microglia, play a vital role in the brain's defence against harmful invaders and help maintain a healthy brain environment. However, in dementia, these immune cells become overactive or not functioning properly. This contributes to inflammation and damage in the brain. By studying the immune cells, researchers can gain insights into how these cells go awry in dementia and potentially find ways to control their functions to protect the brain. Moreover, the blood-brain barrier (BBB) is a protective barrier that separates the bloodstream from the brain tissue. It acts as a gatekeeper, allowing only essential nutrients to enter the brain while keeping harmful substances out. Damage of the BBB is common in many forms of dementia, including AD and VaD. When the BBB becomes leaky or compromised, toxins and inflammatory molecules can enter the brain, further damaging brain cells. Understanding how and why the BBB breaks down in dementia is essential for developing strategies to strengthen and protect this barrier.

Many things can increase one's chance of developing the disease, known as risk factors, including genetics, ageing, obesity, metabolic diseases (e.g., diabetes, cardiovascular diseases, hypertension) and infection. In clinical observation, a combination of risk factors that occur in a person at the same time may change the immune responses and damage blood vessels in the brain. Our research team has been focusing on how these risk factors, including APOE4 carrier, ageing, and obesity-induced diabetes, change the immune cells and blood vessels in the brain that eventually cause AD and VaD. In this proposed project, we will develop different mouse models that have a combination of these factors and study the linkage between the risk factors and changes in immune responses and vessels. We will also use these mouse models to identify diagnostic targets and potent preventive and treatment methods in order to combat AD and VaD. Lastly, we will examine the efficacy of combining newly identified drugs with the current treatment (amyloid antibodies) to advance the AD treatment strategy.

In summary, studying immune cells microglia and investigating how the blood-brain barrier is damaged in dementia is critical for advancing our knowledge of the disease and developing effective treatments. These aspects are like pieces of a puzzle, and by understanding how they contribute to dementia, researchers can work towards finding ways to slow down or even prevent the progression of this devastating condition.

What outputs do you think you will see at the end of this project?

The findings will be published, focusing on understanding the early events of Alzheimer's disease (AD) and vascular dementia (Va) using unique mouse models.

The project will identify therapeutic targets and invent potential treatment methods that could lead to new patents and applications in clinical studies.

Who or what will benefit from these outputs, and how?

In the short term, this project aims to confirm if metabolic diseases e.g., type 2 diabetes and cardiovascular issues lead to immune responses and vascular inflammation that contribute to dementia. Special mouse models will be created to mimic different metabolic conditions, providing a new tool for dementia research.

Human samples from the UK Biobank are limited to fluids (e.g urine, blood,) or genetic data with no brain specimen. Post-mortem brain samples are not suitable for studying the progressive change in the brain. Therefore, these mouse models can help study both late-onset Alzheimer's disease (AD) and vascular dementia.

Looking ahead, in the medium and long term, studying the immune responses and blood-brain barrier can identify targets for better early diagnosis and treatment of late-onset AD and vascular dementia. The mouse models can replicate specific features in human AD brains, making them valuable for discovering potential treatments and drug testing.

Additionally, these models are useful for assessing the effectiveness of newly approved treatments, such as amyloid antibodies.

How will you look to maximise the outputs of this work?

The research findings obtained from the investigation of microglia and blood-brain barrier functions in a late-onset Alzheimer's disease mice model hold great promise in advancing our understanding of the disease and potential treatment strategies. To ensure that the benefits of this research reach a wider audience, we have a comprehensive dissemination strategy which includes but not limited to:

- Publication in Peer-Reviewed Journals:
- Conference Presentations
- Engaging with the Alzheimer's Research Community
- Press Releases and Media Outreach:
- Open Access Databases:
- Collaborative Partnerships with pharmaceutical companies, medical institutions, and research organizations

Species and numbers of animals expected to be used.

- Mice: 6000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are commonly used as animal models in scientific research, including studies on aging and age- related diseases like dementia, for several compelling reasons.

When it comes to aging research, aged mice are particularly valuable because they allow scientists to investigate the effects of aging on various physiological processes and to develop potential interventions. Mice are used as animal models in aging studies because:

- High Genetic and Physiological Similarity between mice and humans
- Well-developed methods of behavior and pathophysiological studies in mice High level of reproducibility
- Genetically Manipulability for specific gene study

In this project, mice carrying different risk factors (e.g. genetics + obesity) from the young adult stage to the old stage will be chosen to study the early pathophysiological changes. A wide range of life stages is chosen as it is relevant to human aging. Age-related changes and diseases take time to manifest. We will employ this wide range of age groups to study how the risk factors change the immune responses and blood vessels during the early pathogenesis, and how these changes lead to the development of AD and VaD in older age groups. Aged mice are essential for conducting long-term studies that closely mimic the chronic nature of conditions like dementia. This makes them suitable for investigating late-stage aging conditions like dementia. Moreover, aged mice can be used to assess the effectiveness of potential interventions or treatments for age-related diseases.

Researchers can study how these interventions impact cognitive decline and other age-related changes.

Typically, what will be done to an animal used in your project?

<u>To develop mouse models of AD and VaD</u>:

We will induce obesity and diabetes by treating transgenic mouse models with a high-fat diet chronically (6 months to 12 months). Mice fed with a normal diet are the control groups. Aged mice will be kept up to 24 months old.

About 10% of mice will also be injected with immune stimulants to mimic the combined risk conditions in dementia.

About 10% of mice will be induced with vascular dementia by surgically narrowing blood vessels under anaesthesia.

For mice aged over 15 months, mice conditions (e.g. blood pressure) will be monitored frequently (at least once per week) by non-invasive device (e.g. telemetry or tail cuff sensor) to ensure animal welfare.

<u>To test potential drugs for treating AD mice</u>:
The mouse models generated above will be treated with drugs by intraperitoneal injection, tail vein injection or oral feeding from 2 weeks to 3 months, depending on the type of drug). A combination of drugs may be tested, meaning that the mice may be treated with more than one drug using different administration routes.

<u>Analysis</u>:
Mice will then have behaviour tests and imaging under anaesthesia.

Mice will be killed humanely by schedule 1 procedures for brain tissue collection.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice will become obese after high-fat diet administration. High fat diet/Western diet are expected to cause a greasy coat (100%), which may lead to over-grooming (~25%) and as a result, possible skin inflammation/ulceration and infection (<5%). If the level of obesity becomes profound, the animals may become less active and a small proportion (1-2%) may experience difficulties in grooming certain regions. This however is not expected to affect the health and well- being of the animals.

A portion of mice will be aged until 24 months and may develop memory deficits.

Aging mice may develop high blood pressure.

Mice that have undergone surgery to narrow the carotid vessel may have a slight weight reduction (usually less than 5%) in the first two days after surgery. These mice will develop vascular dementia under chronic reduction of blood flow due to narrowing of the carotid blood vessel. These mice will eventually develop memory deficits.

Mice undergoing behaviour tests may have mild stress during the test but will be recovered after the test. For mice undergoing Water Maze tests may experience moderate stress during the test but will be avoid with escalated handling and post-test measurements, to reduce stress during and after the test. Mice will usually be recovered quickly after the test.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The adverse effects are considered mild and moderate throughout the lifetime and during experiments. 75% of the mice will experience mild severity. 25% of mice will experience moderate severity.

What will happen to animals used in this project?

- Used in other projects
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Dementia is an age-related cognitive disease involving complex cell-to-cell and tissue interactions. The complexity of regional interactivity in the brain is such that in vitro approaches are inadequate to mimic, as cells in culture dishes cannot maintain full

anatomical and functional connectivity. This approach would fail to express the cognitive features that drive behaviour. The interactions between the brain and the periphery tissues e.g., hormonal control, blood-brain-barrier functions, and immune cells between the brains and the periphery that underlie the development of dementia can only be assessed adequately in vivo using animal models.

Which non-animal alternatives did you consider for use in this project?
There is a case for in vitro approaches that use brain slices for pharmacological screening, which actually becomes advantageous. Brain slices from one brain can help reduce the number of mice and refine the welfare. In another in vitro model, primary cells may also serve for the purposes of molecular studies within one cell type.

Moreover, some primitive and non-protected models, such as zebrafish larvae and drosophila, have been considered as an alternative, but it is impossible to induce obesity in zebrafish larvae. Zebrafish larvae are too early in the life stage, so they cannot be used as a model for studying aged-related disease.

Human tissues including post-mortem sections from patients only allow studying the disease pathology at the end-stage. We cannot study pathogenesis in the onset of the disease.

Computer Modelling and Simulation: When applicable, computer modelling and simulation techniques can be employed to predict outcomes and test hypotheses without the need for live animals.

Computational experiments and modelling can reduce the need for in vivo experiments as a first screening of drug effectiveness. This can reduce the number of potential drugs for animal tests.

Why were they not suitable?

In the project, we will use these culture models to replace the use of animals in some experiments. However, these models cannot replace using mice to study age-related diseases. These in vitro models cannot replace the study of the interaction between the periphery and the brain, as well as the association between metabolic disorders and dementia. Computer modelling can only be used as a preliminary study but cannot replace the use of animals in drug testing.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We will ensure that we use the minimum number of animals through careful design of studies, minimal animal handling by researchers to reduce stress, making sure that

animals are accustomed to any testing arena before a study begins, and providing good researcher training. We will monitor the reliability of our studies closely and alter group sizes as appropriate and in consultation with statistical experts. We are working closely with colleagues to develop behavioural tests that improve data yield to reduce animal numbers further by minimising the potential negative effect of animal handling in our studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?
During the experimental design phase of this project, several steps were taken to reduce the number of animals used, aligning with the principles of Reduction (one of the 3Rs) and ethical considerations. We will make use of:

- NC3Rs Experimental Design Assistant
- Statistical Analysis Plan and Biostatistician Consultation
- Optimal Experimental Techniques
- Sample Collection and Sharing

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In addition to good experimental design, several measures will be implemented to optimize the number of animals used in the project, ensuring that animal welfare is prioritized while minimizing the overall number of animals involved. These measures include:
Efficient Breeding and Colony Management
Use of Pilot Studies
Computer Modelling and Simulation
Sharing of Tissue and Data
Adaptive Study Design for the modification of sample sizes and research protocols based on interim results.
Use of different Non-Invasive Techniques such as imaging, behavioural observations, or remote data collection, allowing different analysis on the same batch of mice

Longitudinal study of small cohort to gather preliminary data before large cohort.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

In this study, mouse models of dementia will be used due to their genetic similarity to humans and ease of genetic manipulation. The methods will emphasize non-invasive behavioural assessments and cognitive testing. Minimization of distress and pain will be

achieved through careful experimental design, such as habituation to testing procedures and the use of positive reinforcement techniques.

Behavioural assessments will be non-invasive and refined to minimize distress.

Observations will focus on natural behaviors, and any potentially distressing procedures will be avoided or conducted with the utmost care and consideration for animal welfare.

Training will be provided to staff and students who will perform oral gavage, intravenous and intraperitoneal injections of testing drugs, and anaesthesia/analgesia with proper techniques to minimise stress and suffering.
Transfer of the mice into the testing arena will be done with the cupping technique to minimise distress. Mice will also be handled by the same person for at least 7 days before behavioural tests are performed. When required, appropriate anaesthesia and analgesia for any invasive procedures will be strictly followed to minimize distress and reduce pain.

Why can't you use animals that are less sentient?

Using less sentient animals or animals in immature life stages, such as embryos or neonates and zebrafish larvae, may be scientifically unsuitable for dementia research.

Dementia primarily affects adult or aged individuals. Therefore, using animals at more immature life stages with significantly different physiology and brain development may not accurately model the condition. Research findings may not translate to the understanding and treatment of dementia in humans. Drosophila is a good model for studying the basic biology of neurodevelopment and protein functions but not for age-related and pathological studies. Using animals that are not biologically relevant to the research question can lead to inconclusive or invalid results. Scientific rigor and validity are crucial in dementia research to ensure that findings can be extrapolated to human patients. In dementia research, it is crucial to strike a balance between the ethical treatment of animals and the scientific validity of the results. Using appropriate animal models, typically adult or aged animals, ensures that the research is ethically conducted and that the findings have practical relevance to understanding and treating dementia in humans. Researchers are encouraged to adopt the highest ethical and scientific standards to pursue this important research while minimizing harm to animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To achieve this, the following steps can be taken:

Review existing procedures to estimate the potential sources of stress, pain, or discomfort for the animals.

Pilot studies to identify potential issues in the procedures.

Use of anesthesia and analgesia: to minimize pain and distress during procedures.

Explore alternative techniques that are non-invasive to minimize the need for invasive procedures.

Refine experimental protocols to identify opportunities for improvement e.g., adjusting dosages, refining timing, or minimizing the duration of procedures.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow guidelines and recommendations from professional organizations associated with the specific field of research, such as the Laboratory Animal Science Association (LASA) and the Animal Welfare and Ethical Review Bodies (AWERB 2.0), to ensure the experiments are conducted in the most refined way. We will also refer to published guidelines of substance administration by Jenning M. et al., (doi: 10.1258/la.2008.007143.), and the NC3Rs.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

To stay informed about advances in the 3Rs (Replacement, Reduction, and Refinement) principles and implement these advances effectively during the project, the following strategies will be employed:

Regular Literature Review on the latest research articles, publications, and scientific journals related to the 3Rs principles.

Participation in Workshops and Conferences focused on the 3Rs provides a platform for learning about cutting-edge techniques, technologies, and ethical considerations in animal research.

Consultation with Ethical Review Boards e.g., AWERB

Engagement with Regulatory Bodies with updated guidelines and regulations to reflect the latest 3Rs principles and compliance with the updates.

15. Tissue level dynamics and structure drive loss of function in ageing

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Ageing, Extracellular matrix, Tissue regeneration, Tissue organisation, Protein translation

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand how tissue level changes, for example in the extracellular matrix (ECM) or global translation and metabolism, drive ageing in tissue. Ultimately, we aim to discover how to reverse those changes in tissue to return optimal function to tissues in ageing and age-related disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Though we have seen a massive increase in lifespan over the last 100 years due to

advances in medicine, there have been relatively fewer advances in tackling age-related diseases, and many people experience significant morbidity as they age. This is becoming an intense societal problem due to our ageing population. Many have studied age-related disease at the cell level, but we believe that ageing happens at the tissue level, and particularly changes the function, structure, organisation and mechanical properties of the tissue. However, little is known about what changes are actually occurring, and which ones matter. Thus, we intend to identify how ageing progresses at tissue scale, and how we can reverse those changes, in order to develop better interventions for age-related morbidity and disease.

What outputs do you think you will see at the end of this project?

In the short term, we will be providing two major outputs in the form of publications and data resources. The work performed under this project licence constitutes novel basic research which will lead to new insights into how ageing at the tissue level leads to loss of organismal function. Also, we will be providing tissue level readouts of how structure, composition and mechanics change with age, which will be important datasets for the scientific community. In the longer term, the insight we gain on tissue level ageing and possible interventions will be highly translatable and will be used as the foundation for therapies to treat age-related disease.

Who or what will benefit from these outputs, and how?

In the short term, the scientific community will gain most from our research. Our research will provide new insight into ageing of tissue and how that contributes to age-related loss of function and age- related disease. In the longer term, our translatable work could be used for new therapies to alleviate age-related tissue morbidity. In this case, the benefit would be society-wide.

How will you look to maximise the outputs of this work?

We will publish our work and make all of our datasets available to the public (once relevant IP has been established). We will publish both successful and unsuccessful approaches and discuss our work on scientific conferences and meetings. We will also be working within the organisation to turn our work studying interventions in tissue-level ageing into therapies.

Species and numbers of animals expected to be used

- Mice: 19830

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

<u>Mice are the most appropriate model system for this project for the following reasons:</u>

Similarities in structure and cell behaviours in tissues of interest, for example the skin. Mouse models are well published as a brilliant model for skin studies.

Similarities in the ageing process: Mouse models recapitulate many of the key detrimental age- associated effects seen in human ageing.

Available tools: Mouse models with vital genetic tools already exist and are well validated, with harmful effects minimised.

Life span: The mouse life span allows for ageing studies in a more timely manner than that of other rodent or non-human primate species.

Breeding: Breeding and maintenance of mouse colonies provide optimal litter sizes for well controlled experiments in a timely manner, minimising colony size and animal usage.

Non-mammalian models do not possess the required similarities to human tissues and ageing pathways.

To achieve the objectives set out in this project, the use of animal models is unavoidable.

Mouse systems allow for a combination of required animal work and paired cell-based studies.

<u>We propose to use the life stages outlined in this application for the following reasons:</u>

Embryonic:
The activation of genes during the early stages of life is needed to understand their impact throughout an organism's life.

By inducing this activation when the organism is still in its embryonic stage, we can conduct studies that cause less discomfort to the animal and shorten the time it takes to reach ethical endpoints.

Initiating experimental procedures during the embryonic stage allows for the collection of conclusive data, reducing the need for additional groups of animals.

Adult:
We need to understand how tissues in the body are maintained in a balanced state (homeostasis) to investigate problems arising from dysregulation of the balancing act.

In this project, adult time points will serve as the 'control' in most studies.
Based on published research and industry standards, scientists typically consider adulthood as the stage at which interventions are expected to address age-related decline.

Aged:
Aged mouse models play a crucial role in this research, as they are essential for understanding the harmful changes that happen to tissues as organisms age and how these changes can be reversed or reduced.

Aged mice are the most commonly used models for studying aging in living organisms, and the work must be reproducible, reliable, and possible to share with other researchers.

The processes of aging in mice closely resemble those in humans.

Typically, what will be done to an animal used in your project?

Typical experience example 1:
Genetically altered animals will be selectively bred to create a strain of interest. When these animals are adults, they will be weighed and given a single injection into the abdomen to turn on a genetic marker.

Injected animals will then be kept for one week. During this week, a cream may be applied to the back skin of the animal to change the way skin cells behave. At the end of this week, the animals will receive one more injection, under the skin, to label all of the cells that are proliferating at that time, before being humanely killed.

Typical experience example 2:
Genetically altered animals will be selectively bred to create a strain of interest.

When these animals are developing embryonically, the mother will be weighed and given a single dose of a compound orally to turn on a genetic marker. The resulting pups will have the genetic marker turned on from early development throughout their life, and so these animals will be kept to adulthood before being humanely killed.

What are the expected impacts and/or adverse effects for the animals during your project?

Expected impacts and/or adverse effects are as follows:
All injections will be given via typical routes, as used in humans. Similarly to in our case, a pin prick will be felt by the mouse and some irritation can occur in the site immediately after injection. This will pass quickly and leave no lasting damage at the site.

As much as possible, drugs given to the mice will have been tried and tested in animal models before. We will use recommended doses as typically seen when calculating how much of a drug to give to a person. Where this is not possible, we will use published data, titration studies and advise from colleagues in the field to establish safe dosing.

Wounds inflicted on mice will be relatively small and should heal back up completely within approximately 10 days. Mice are expected to recover quickly and will be given painkillers and post- operative care just like people recovering in the hospital or at home from a minor injury.

Live imaging of animals will be done in a way that does not cause unnecessary distress to the animal by using anaesthetics to put them to sleep for the duration. Similarly to in surgery with humans, the mice will be woken back up post-imaging and given post-operative care just like people recovering in the hospital.

Transplantation will be carried out in a very similar way to that described in part (3). Mice are expected to recover quickly and will be given painkillers and post-operative care just like people recovering in the hospital or at home from a minor injury. They will be monitored very closely.

Ageing of mice will only be done when absolutely necessary for the experiments. To improve the quality of life and enrichment, we will do our best to make sure that mice are house in groups and provided with environmental enrichments. Extra care and observations will be provided to ensure that typical age-associated decline is not having too great of an effect on animal welfare.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice: 10% Sub-threshold, 43% Mild, 47% Moderate.

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Ageing, including associated diseases such as cancer and inflammatory syndromes are complex and often severe conditions. To understand the fundamental molecular mechanisms and develop rational therapeutics, clinical, behavioural and pathological outcomes, experimental models are essential.

Whilst invertebrates are in many ways different from humans, mice share many similarities. Biochemically and physiologically many of the mechanisms, processes and pathways are identical to those in humans. Furthermore, the availability of genetically altered animals with gene knock-outs or over expression enables us to elucidate the importance of particular proteins in ageing and associated diseases, and to extract material for cell-based systems.

Which non-animal alternatives did you consider for use in this project?

As a part of this project, we will utilize 3D cell-based culture systems like organoids or epithelioids. These systems mimic the structure and various cell types found in different tissues inside the body. This will allow us to manipulate gene expression, treat with drugs, place them in different environments and study dynamics. These cell-based experiments will help to understand the wiring of molecular networks, cell behaviour and how these can go awry in ageing and disease. This project includes an important tissue culture component, representing a significant replacement of techniques requiring animals wherever possible. Use of more complex cell-based models such as 3D organoids requires collection of material from animals, but greatly reduces the number of animals required.

Why were they not suitable?

Development of ageing and associated diseases requires months to years and renders cell-based approaches non feasible due to the limited lifespan of cultured models. Whilst cell-based models have many of the same features observed in animals, the complexity of interactions between the cells and their surrounding environment together with cross-tissue interactions means that there is ultimately no substitute for animal experimentation.

Key genes and pathways identified will require validation in order to demonstrate that they operate in animals as well as in the cell-based environment. This is essential to enable translation of findings into potential therapies to treat disease.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Estimated numbers were calculated based on ideal breeding outcomes from GA lines of interest and attaining the desired cohort of animals with minimal breeding stages. We have made estimates based on advice from those with great experience in animal work, particularly named persons, to design optimised breeding and maintenance strategies.

Due to the spread of time-points of interest in this project, we endeavour to utilise as whole litters wherever possible, using littermate controls, for example.

We have also taken into account that many aims and objectives can be addressed in multiple tissues of interest from the same experimental animal, minimising repeated experiments. As such harvesting of multiple tissues from individual animals will be a priority throughout.

Finally, estimated numbers have also been based upon experience with similar experiments and understanding of required numbers to achieve scientific accuracy.

Advice and guidance has been sought after wherever we did not have accurate data indicating approximate animal usage for particular techniques.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have taken a range of steps during experimental design and planning to reduce the number of animals being used in this project, as listed below:
Desired GAAs, crosses and inductions have been selected to work in a range of tissues of interest to allow multiple aims and objectives to be addressed with each experiment. We hope that this planning will mean that very few, if any, animals will be humanely killed for the collection of only one tissue.

Each protocol is written in a way that allows for adjustment for changing experimental needs as we gather data. To this end, we have included consistent optional steps in many protocols so that multiple aims can be addressed in individual animals. For example, by including both transgene induction, cell labelling and a variety of sample collection methods, we plan to minimise the number of unique experiments.

We have discussed within the group who will be working on this project licence to ensure that all needs can be met with similar experimental designs. This includes an established plan for animal numbers needed for specific experiment types, such as looking at qualitative or quantitative aspects and the requirements of reliable replicates of each.

We have researched and validated appropriate cell-based experiments, matched to each tissue of interest to allow for further reduction in the number of animals being used in this project. For example, we have verified the applicability of epithelioids in replicating features of interest in studies of the epidermis. This will allow multiple experimental set ups to be tested in cell-based assays from individual animals. In the context of epithelioids as just one example, this allows the use of back skin from one animal to be split into approximately 48 individual epithelioid cultures.

Pilot studies have been, and will continue to be, designed in a way that will produce usable data from the start point. We aim to use literature and experience to validate pilot studies as much as possible.

SOPs for each protocol will be established to ensure reproducibility and reduce the numbers of required animals. This will control for aspects including time of day dosing, method of drug formulation and storage, handling, diet and bedding.

Tissues will be shared at any point possible. We have also reduced our estimated number of animals to account for possible tissue sharing from other projects and collaborators.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Optimisation of animal usage will be achieved using the following measures:
Breeding and maintenance controls. Minimisation of unnecessary crossing and breeding by carefully selecting breeding pairs or trios for optimal outcomes. This will be done based on advice and experience from the named persons within the facility and based on literature. Using tightly controlled mouse lines that can be turned on and off as needed, and making sure that all their descendants have the same genetic makeup, ensures that all the mice born from this line can be used for experiments when necessary.

Pilot studies have been, or will be, designed to follow the intended experimental design, enabling use of pilot studies results as experimental data wherever possible.

Data analysis, computer modelling and statistics are a strength within the group and will be used to ascertain the minimum required number of animals for each experiment to achieve scientific confidence.

Aligned goals within the research aims will allow for optimal use of animals by taking several tissues with every collection.

Tissues will be shared at any point possible. We have also reduced our estimated number of animals to account for possible tissue sharing from other projects and collaborators.

Cryopreservation will be used to preserve important lines and remove the necessity to hold livestock for extended periods.

We will share our animal lines with other researchers and place them in international repositories, where appropriate, in order to reduce the number of animals used globally to derive these lines.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Models:
The use of genetically modified animal models can be split into different categories depending on the intended use:
(i) Cell-specific labelling or lineage tracing involves experiments where animals typically carry a CRE gene (either tamoxifen-inducible or not) driven by a specific promoter (like ubiquitous Rosa26, Krt14 for labelling basal cell progenitors or Lgr5 for labelling intestinal stem cells, etc.). These animals also have floxed alleles that, when they undergo recombination, lead to the production of a tagged protein (RPS22-Flag), a fluorescent protein (e.g., tdTomato), or an enzyme (LacZ or MetRS, which is a modified methionyl-tRNA synthase that allows for the incorporation of amino acid analogs into new proteins). These models have been extensively used and tested, and they provide strong and dependable results without causing significant harm or distress to the animals, minimising any pain or suffering.
ii) Disease models, loss, and gain of function models are very useful in understanding ageing. We have special mice with specific mutations, where some genes are turned off or turned on more than usual. These mice help us test how important certain genes, signals, and pathways are in ageing, both in animals and in lab-based experiments with cells. For instance, we have models for diseases like Ehlers-Danlos syndrome, rapid ageing (like Hutchinson–Gilford progeria or RPS9 mutants), and diabetes (like the db/db mouse). We also have models where we disrupt specific pathways, such as the mTOR pathway with Raptor or Pten mutants. We even have mice with normal or altered proteins that can make animals live longer. Whenever we can, we use these special mice in a way that only affects certain cells or tissues, which helps us learn without causing the animals too much pain or suffering.

Methods:
We will use validated methods from previous work and publications wherever possible, and

be adaptable to further refinement opportunities that may arise.

In addition we will implement the following methods for minimising suffering: (i) performing all surgical procedures under general anaesthesia, (ii) administering disease modifying agents/labelling compounds/transgene regulators with due care and pain relief if required, (iii) staged dosing, (iv) adhering to published guidelines, (v) use of the most refined compounds possible, with minimal adverse effects, (vi) use of ultra-fine needles where possible to reduce damage at injection sites, (vii) housing mice in groups with enriched environments as much as possible, (viii) minimising the number of surgical interventions.

Statistical planning will minimise animal usage. For ageing mice, we will use body-condition scoring (using standard published criteria) as well as other regular checks to accurately monitor the ageing process and define humane endpoints thereby reducing suffering.

Why can't you use animals that are less sentient?

Wherever possible, we will use animals at a more immature life stage. However, due to the nature of this ageing project, it is required to work with adult and aged animals.

Less sentient animals show less similarities in their aging process. Overall, mice are the least sentient model system we can use that has relevant physiology and tissue complexity, while being amenable to genetic manipulation.

Where relevant, tissues will be collected from humanely killed animals that have not undergone any procedures, or collected as part of a tissue sharing initiative with other researchers.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Beyond the legal expectations (performing all surgical procedures under general anaesthesia, administering disease modifying agents/labelling compounds/transgene regulators with due care and pain relief if required), we will implement the following methods for minimising welfare costs: (i) staged dosing, (ii) adhering to published guidelines, (iii) use of the most refined compounds possible, with minimal adverse effects, (iv) use of ultra-fine needles where possible to reduce damage at injection sites, (v) housing mice in groups with enriched environments as much as possible, (vi) adaptable weighing and monitoring scheme to allow for increased needs in ageing animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will be using the following published best practice guidance:
PREPARE Guidelines
NC3R Guidelines
NC3RS Experimental design assistant

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We endeavour to remain adaptable to advances in techniques. As such we aim to remain up to date with information and talks from NC3Rs and will ensure regular updates are shared with all licence holders. A 3rd party contract research organisation that will house the mice will also be providing regular updates.

All experiments will be regularly assessed with respect to the 3Rs.

16. Genes and lifestyle influencing growth in the womb and lifelong health

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Brain health, Genomic imprinting, Healthy ageing, Metabolic health, Obesity

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to discover mechanisms influencing growth and development in the womb that affect size at birth and the proportions of different tissues such as muscle and fat. We will test the idea that these early changes can be influenced by adverse circumstances, such as poor maternal diet during pregnancy, and can have lasting effects on diverse health outcomes.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Birth weight is a universal health indicator, yet the influence of genes and environmental factors on the establishment of tissue proportions is rarely considered.

Early changes in the development of muscle and fat tissue may have lasting effects on fat accumulation and the risk of developing obesity and diabetes, as well as on muscle mass

across the lifespan, influencing age-related muscle loss (sarcopenia).

Similarly, altered brain development may increase the risk of common mental health disorders such as autism and age-related conditions such as Alzheimer's. Our basic research is designed to uncover new mechanisms contributing to highly prevalent societal health problems that could lead to novel interventions in the future. In addition, several genes we study have causal roles in rare growth disorders such as Beckwith-Wiedemann and Silver-Russell syndromes. Our work will improve understanding of all these conditions and is likely to lead to new diagnostic tools and treatments that could be simple dietary interventions or supplements during pregnancy in the case of establishing healthy tissue proportions during pregnancy.

What outputs do you think you will see at the end of this project?

The main outputs will be new information, scientific research papers, and presentations at scientific meetings. Where possible findings will be disseminated to the scientific community and the public in talks, articles in news media and social media announcements.

Who or what will benefit from these outputs, and how?

The main beneficiaries in the short term will be other scientists in fields including developmental biology, genetics and epigenetics, adult physiology, metabolism and brain function. Our work will be of interest to those working on aspects of health and disease relating to these fields, such as obesity, type 2 diabetes, sarcopenia, autism and healthy ageing. Several scientific papers are likely to arise during and shortly after the duration of the project. In the longer term, information may be translated into new diagnostics, to identify vulnerable groups, and interventions such as dietary advice or supplements in pregnancy to protect against common health conditions of later life. Knowledge will be disseminated to students and interested members of the lay public.

How will you look to maximise the outputs of this work?

During the project we will collaborate closely with other groups, including those working on human populations and with patients to exchange the latest findings and ideas. We will present our findings at scientific meetings to encourage knowledge exchange at an early stage and engage as widely as possible with the scientific community. We will publish in open access journals and provide the earliest possible access to our discoveries by making our work available first on preprint servers such as bioRxiv and Europe PMC. Where possible we will publish negative findings and unsuccessful approaches so that others will avoid repeating the same experiments.

We are committed to making available to other researchers our data, animals, tissues and other biological agents that we generate. These resources will be made available immediately to collaborators and at the point of publication more generally.

Species and numbers of animals expected to be used.

- Mice: 5,490

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our project investigates growth and development at the level of tissues, organs and the whole body in a manner that is currently impossible to reproduce without studying the whole animal. Specifically, our study involves determining the impact of genes and the maternal environment on body size and tissue proportions during development, requiring the study of fetal stages. In addition, we will link the developmental effects on fetal growth with long-term effects on body composition and diverse health outcomes, requiring the study of animals at young and old post-natal ages. Developmental programming of adult health and disease is essentially specific to mammals, due to the characteristic way offspring are provisioned during development by maternal resources via the placenta and mammary gland. The involvement of imprinted genes is also unique to mammals. The mouse is the model of choice for two main reasons: i) The range of data resources, strains and techniques available for the discovery of gene function is unparalleled among mammalian model species. Lately, this includes detailed single cell transcriptomic 'atlases' of mouse development, including throughout organogenesis. ii) Fetal growth mechanisms and the link with health outcomes in later life is highly conserved with the same processes in humans.

Typically, what will be done to an animal used in your project?

In our purpose-built biological services unit, mice will be group housed in individually ventilated cages under standard conditions of 13 hours light and 11 hours darkness, including 30-minute periods of dim lighting to provide false dawn and dusk, a temperature of 21±2°C and relative humidity of 55±10%.

Cages will contain environmental enrichment and mice will normally have access to food and water ad libidum. Experimental litters will be generated through natural matings. Mice will be studied at embryonic or fetal stages using genetically altered strains, sometimes following manipulation of the maternal diet prior to and during pregnancy. In some cases the pregnant female may be administered with substances (typically once by injection or via drinking water) that induce gene expression at specific stages or in specific tissues, for the labelling of specific cells or cellular components (e.g. dividing cells), or to modulate signalling pathways (e.g. receptor agonists, such as growth factors, or small molecule inhibitors of receptors or other signalling molecules). Otherwise, dams will be humanely killed, and all further analyses of dam and fetal offspring will be conducted post-mortem.

Some of the GA strains we study are known to have homozygous lethal phenotypes.

When crosses are set up that could contain such offspring, analyses are restricted to stages up to the day of birth. Maternal *Grb10* KO allele females are monitored for failure of the vaginal opening to appear (which normally occurs by 4-6 weeks of age) so that they can be humanely killed prior to visible signs of uterine oedema.

Post-natally, mice may be weighed from birth to adulthood. Neonates may be fostered to

nurses with different genetic make-up or dietary exposure during pregnancy to assess pre- and post-natal effects on offspring. Offspring will usually be marked by ear-clipping at around 3 weeks of age, but where it is necessary to track the weight of individuals from earlier ages, they may be marked by tattooing of paws. From 3 weeks onwards animals will typically be weighed at weekly to monthly intervals, and less frequently subject to NMR scans for analysis of lean and fat body composition (typically 3 to 6 monthly intervals). The NMR scans require animals to be placed in a restraining tube, typically for less than 2 minutes in total. Mice may be implanted with a telemetry device to enable subsequent non-invasive measurements (e.g. temperature). The same mice may be subject to further tests, including: i) glucose and insulin tolerance tests, typically once or twice each during their lifespan (involving injection of fasted animals with insulin or glucose and removal of a few microlitres of blood from the tail vein at several time-points over the subsequent 2 hours); ii) blood pressure measurements using a non- invasive tail cuff procedure, typically at monthly or longer intervals (includes repeated daily training measurements over a few days to acclimatise animals to the procedure); iii) measures of muscle strength (e.g. using a grip strength meter) at monthly or longer intervals. In some cases, mice will undergo one or more of the tests in combination with being: iv) injected with substances such as growth hormone to mimic therapies used in people with growth deficiencies; v) subject to dietary manipulation, typically high-fat or high-fat and high-sugar over several weeks. Animals may be isolated for up to 14 days to enable measurement of food intake. Juvenile or adult mice may be injected with substances to enable regulation of transgenes, short term labelling of cells, or to modulate signalling pathways. Cohorts of animals may be kept into old age to provide measures of ageing and the healthy lifespan. These mice will be carefully monitored using a surveillance regime that we have successfully implemented previously to forestall any end-of-life suffering. In some instance, animals under non-recovery anaesthesia will be perfused with substances such as fixative to preserve tissue for histology. All other analyses will be conducted post-mortem.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of animals will be studied at fetal stages following humane killing of dam and offspring. Dams may be subject to dietary manipulation prior to and during pregnancy and in some cases one or a few injections during pregnancy.

Experiments are typically designed such that dams are subject to a regular (control) diet and one or more test diets in equal proportions. Likewise, offspring kept into post- natal life will usually be equally divided to receive regular and test diets. At the same time the genetic status of the dam may be altered (typically through presence or absence of a specific gene or allele) and offspring will usually vary in their genotype. This could, for example, include animals that inherit an active or inactive copy of an imprinted gene (1 to 1 ratio among offspring), animals homozygous for mutant non-imprinted gene (1 in 3) or compound mutants where the rarest genotypes may be represented at lower frequencies (typically 1 in 8).

Depending on the specific experiment, offspring will usually be studied for several months (some for years, to assess effects on healthy ageing) and subject to multiple tests, each of which is mild in severity. Tests will be spread out to minimise additive stress on animals but the cumulative harm on mice in these adult cohorts will be moderate overall.

Animals kept to a healthy ageing endpoint may suffer transient moderate harm and will be

humanely killed at the first indication of excessive weight loss or other signs of frailty, including solitary, circling, hunching or piloerectory behaviour, or the detection of a visible or palpable mass that could be cancerous. Mice subject to altered diets may lose weight (nutrient poor diets) and will be returned to a regular diet or humanely culled before they lose 20% of bodyweight. Mice provided with nutrient rich (high fat and/or sugar) diets are intended to gain weight and may become obese. In both cases the changes in body weight may be deemed moderate in severity.

Most animals will be studied at fetal stages following humane killing of dam and offspring. Offspring studied at post-natal stages will typically be kept for up to 9 months of age with a minority kept for around 48 months to study healthy ageing.

Mice may experience weight loss or gain as a consequence of genetic manipulation or diet. This may include dams prior to and during pregnancy as well as their offspring. The effects on weight are unlikely to cause major harm but may necessarily last for several months. Food withdrawal for fasting periods, used prior to glucose and insulin tolerance tests, will usually be kept to around 6 hours (maximum 8 hours), mainly during the hours of light when mice are already less active and feed less. Isolation of animals, for example to determine individual food and water intake, will be kept to a maximum of 14 days. Any other adverse effects will be transient, such as pain associated with injections or blood sampling (seconds), discomfort while subject to body scanning (minutes) or mild stress associated with testing of blood pressure or muscle strength. Rarely, cross-fostered litters may be rejected by the nurse, otherwise no adverse effects are expected that are inherently due to the act of cross-fostering. Animals subject to procedures under non-recovery anaesthesia will only be aware of the anaesthetic being administered and may experience mild distress and no pain.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

68% sub-threshold
16% mild
15% moderate
1% non-recovery

What will happen to animals used in this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We are studying the growth and development of the whole organism, including the ratios of different tissues, such as muscle and fat. We aim to discover the effects of genes and the environment during pregnancy that influence growth and tissue proportions, and the underlying mechanisms. Further, we aim to link these developmental changes with post-natal physiology, health and ageing across the life- course. There are currently no alternatives to studying the complex interactions involved in these processes, including the transfer of nutrients from mother to offspring across the placenta and mammary gland, cross-talk between individual organs, and the response to these developmental changes in adult life at the level of tissues, organs and the whole body. These interactions are unique to mammals.

Some of the genes involved in these processes that we study are regulated by genomic imprinting. Imprinting restricts expression to only one of the two inherited copies, in a parent specific manner, and is also unique to mammals.

During the project, where aspects of the GA mouse phenotype can be studied in isolation we will seek to do so using *ex vivo* or i*n vitro* culture models. For instance, we can isolate embryonic fibroblasts and organ rudiments from wild type and genetically altered embryos to study characters such as rates of cell proliferation, survival and metabolic activity. To eliminate the need to use animals as the tissue source we can in principle use cell lines or organoids generated from stem cells. To be of use these will need to be modified by CRISPR/cas9 technology to harbour the same types of mutation we will study in vivo. Ultimately, these models will have limited value until we have greater understanding of the cellular and molecular fetal growth mechanisms. For instance, they are unlikely to accurately represent cells and conditions in the embryo, such as rare and sometimes transient progenitor cell types, interactions between cell populations within and between tissues, or changes in the environment of the womb due to maternal genetic and lifestyle factors.

Which non-animal alternatives did you consider for use in this project?

We have considered the most complex systems available to study mammalian growth other than the whole body. This includes conventional cell cultures, stem cell-derived organoids, synthetic stem cell- derived mouse embryos, non-protected species and human population studies.

Why were they not suitable?

Conventional cell cultures and organoids fail to represent the in vivo situation in which the growth of one tissue or organ may impact that of other tissues. Stem cell-derived synthetic embryos are still very limited in their developmental potential and their generation is far from routine. The current limit for their development is to a stage thought to approximate embryonic day e8.5 (e8.5), which allows researchers to study aspects of gastrulation and the initial events in organogenesis. However, even in real embryos at this stage the rudiments of most organs have yet to be formed and in some cases are not even represented by the earliest recognisable progenitor cells (notably including adipose tissue). The genetic and environmental influences that we will study are predicted to influence precursor populations that emerge at later development stages and will not be represented in synthetic embryos. Further, growth of stem-cell derived synthetic embryos is highly variable and one of the least well controlled aspect of their formation. One major limitation to synthetic embryo growth is the lack of a functional placenta and connection

with the mother. Thus, like all cell and organoid cultures synthetic embryos are sustained by nutrients coming directly from the culture medium and not the mother.

Consequently, mimicking the effects of adverse conditions during pregnancy, such as altered maternal diet or maternal obesity, is currently impossible to achieve. Non-protected species such as fruit flies, or vertebrates considered less sentient such as fish, also lack these mammal-specific interactions between mother and offspring.

Similarly, discovering the mechanisms linking early growth with long term consequences for physiology and health is only possible through animal studies involving mammalian species. While human population studies can demonstrate the same phenomenon, that genetic and environmental influence on both birth weight and health in later life, they have much more limited potential for discovery of the underlying mechanisms due to the limited availability of fetal material and associated ethical barriers.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Essentially three categories of mice will be used in the project. 1) Stocks used to maintain the unique genetic lines required to generate experimental animals.; 2) Dams carrying litters of embryos to be studied at fetal stages; 3) offspring to be studied from birth into adulthood.

Estimates are based on our previous annual returns, accounting for the disruption to our work over the last few years due to the pandemic. We have also considered the numbers of lines we will maintain and assume a reasonable level of funding for the work we propose over the next 5 years. Stocks will be maintained at the minimal levels to safely preserve each line and lines with no further intended use will be kept only as cryopreserved material.

Experimental groups typically compare 2 or more genotypes, one of which is the wild type control, ensuring that litters of experimental offspring are controlled for genetic background and environmental influences. Where treatments of dam or offspring are performed, such as dietary interventions, the allocation of equal numbers of subjects to each diet is randomised. For experimental animals, estimates take account of effect sizes and variability of characters measured in previous experiments, as much as possible. Our estimates also consider the need to take account of effects such as litter size and offspring sex. When studying new growth characteristics or taking measurements at a specific embryonic stage for the first time, a pilot study involving a few litters will be performed to ascertain estimates of effect size and variability.

Using values from previous experiments or pilot studies we have used statistical methods to estimate sample size numbers, which are typically 8-10 per group.

Embryos within and between litters also need to be carefully stage matched for accurate comparison of growth characteristics. This increases the number of litters needed to generate sufficient stage- matched offspring but in turn reduces variability in growth measures, contributing to robust statistical analysis.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We minimise the number of animals necessary using experimental designs that maximise the robustness of the data produced. These designs include minimising variability, operator blinding and mathematical modelling to account for litter-specific differences. We minimise variation in our experiments by using mice with an inbred (C57BL/6) or mixed inbred (C57BL/6:CBA) strain background as standard. The mixed inbred strain background is needed to avoid strain-specific harmful effects known to occur in specific strains we will use. We also reduce variation by routinely studying offspring from previously nulliparous dams, since we have shown that offspring from first and second litters differ in their growth characteristics. For the examination of fetal stages, offspring are collected from dams that have been plug checked and are assigned to a specific stage based on characteristic morphological features. This means some embryos or whole litters may need to be rejected (though we will always seek to repurpose them if possible) to ensure that accurate comparisons can be made between properly matched embryos.

Where dam or offspring are subject to a treatment, such as different diets, they are randomly assigned to control and experimental groups. Analyses, from physiological testing of live animals to post-mortem evaluation of tissue histology and gene expression are conducted with the operator blind to genotype and other information such as dietary treatment. Measurements are subject to rigorous statistical analyses that can include modelling of complex data (e.g. linear mixed modelling and mediation statistics), which we do in collaboration with a statistician. Experimental design expertise of the licencee has been built up for over 30 years. We embrace new ideas and advice that come from diverse sources. Not least we periodically consult the NC3Rs website and liaise with their representatives, the local AWERB committee, ARLO and animal user group within our institution. We have consulted the NC3R's Experimental Design guidance and experimental design assistant (EDA) and ensure that our publications comply with the ARRIVE guidelines.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use efficient breeding schemes to maintain our GA mouse strains and to produce experimental animals of the desired genetic composition. We routinely study males and females as separate groups to identify sex-specific effects. While this increases the total number of animals in an experiment, it makes the most efficient use of animals within each litter and provides more robust datasets. Pilot studies will help us ensure the feasibility of experiments. Computer modelling of previous experiments may help us reduce the number of interventions or sampling time-points needed to achieve a robust experimental outcome. Where it will not compromise the main experimental purpose, we are in the habit of collecting an array of tissues from each experimental animal and processing these for multiple purposes (e.g. fixed tissue for histology together with frozen tissue for extraction of nucleic acids and proteins). When we study the fetus we also collect and study the

placenta. Our experiments are often designed to allow post-mortem analysis of multiple organ systems from animals for which we have extensive physiological data. We often share tissue with other researchers, making use of our tissue archive or surplus stock.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Our work is specific to mammals for the reasons outlined earlier. Among mammals, mice provide the optimal system to combine genetics (for instance, whole genome sequences are available for multiple mouse strains) and gene manipulation (including targeted modifications through ES cell and CRIPSR/Cas9 technologies) with analyses of growth, development and physiology. The involvement of imprinted genes in many of the planned experimental crosses is generally advantageous because the phenotype will be manifest in heterozygotes inheriting a mutant allele from the appropriate parent and will usually be present in 50% of offspring (as opposed to 25% in the case of homozygous recessive alleles, typical of non-imprinted genes).

All Protocols are based on standard, published techniques that adhere to Home Office guidelines and are the most refined techniques currently available to us. We keep up with technical advances such that further refinements can be implemented promptly. When alternative techniques are available priority is given to the mildest appropriate means. We use physiological tests that are robust and standard in the field, which facilitates comparison between experiments, including those carried out in different laboratories. We always seek to minimise exposure to stress, harm or pain (e.g: shortest periods of isolation or restraint), without compromising experimental validity, including use of appropriate administration of anaesthesia and pain relief.

The project involves genetic crosses involving one or more GA mouse strains where the altered genes affect fetal growth and aspects of post-natal physiology. Where the gene alteration causes animals to be larger at birth than normal this does not compromise parturition. In some cases, homozygous GA animals that are small at birth do not survive beyond hours or days post-partum and will only be studied up to the day of birth. GA animals that manifest harmful defects are maintained as carrier stock where possible (e.g. mutations in imprinted genes can be bred through the parent that transmits the normally silent allele), but where affected offspring must be bred, we have in place a monitoring scheme that aims to limit as far as possible the risk of undue suffering. For instance, there is no need to maintain the minority of female *Grb10* KO mice that present an imperforate vagina, since they cannot be used for breeding and the defect could compromise any physiological data due to consequent bloating of the uterus. All potentially affected females are visually inspected carefully between 4-6 weeks and humanely killed if the vaginal opening is not formed by 6 weeks of age, which typically precedes any signs of abdominal

swelling due to uterine oedema.

Some crosses will include GA strains that incorporate reporter genes to facilitate the study of gene expression or strains with driver genes that enable genetic alterations to be switched on or off at specific developmental stages or in a tissue-specific manner. Such reporter or driver genes have no, or negligible, intrinsic detrimental effects. In some cases, driver genes are temporally activated by delivery of an activating substance (e.g. the synthetic hormone analogue doxycycline) and certain reporters require a substrate (e.g. luciferin for luciferase), which again have no appreciable detrimental effects when used at standard doses. Dosing of such substances will be conducted through their addition to food or water where possible, and only by the more invasive injection route where this is necessary to precisely control delivery or where oral administration is ineffective.

Where modified diets are to be used, the composition and effects on mouse body weight are known. For nutrient depleted diets (e.g. low protein) mice are expected to lose less than 10% of body mass. For fat and sugar rich diets mice are expected to become obese (high fat diet) with modest glucose and insulin resistance (high fat and sugar) in comparison with controls fed a standard composition diet. By following published protocols that induce a robust response with the shortest duration on the modified diet we will ensure that our results are comparable to other studies while causing the least harm.

For NMR scans mice are typically held in a restraining tube for less two minutes total, including the scan time, and they do not need to be anaesthetised (avoiding the associated risks and harms). These scans of live animals allow us to obtain estimates of lean and fat body content from the same animals at different post-natal ages. This allows total numbers to be reduced compared to techniques conducted post-mortem, in which separate cohorts are needed for different time points.

Glucose and insulin tolerance tests are long-established standard methods for testing whole body glucose handling. They rely on fasting of animals to set a baseline circulating glucose level, followed by administration of a uniform glucose load and tracing of blood glucose levels over a 2-hour period to monitor glucose clearance from the bloodstream.

We inject the glucose load rather than using the more stressful oral gavage method and have refined the fasting period from overnight (12-15 hours) to 8 hours or less, mainly during the daylight period (when feeding is already much reduced).

Blood pressure is measured using a non-invasive tail-cuff method. This requires some sessions acclimatising animals to the machine before reliable readings can be acquired.

Measurement of body temperature by telemetry involves a single transient instance of pain at the moment of subcutaneous implantation, but means repeated measures can then be acquired without the need for further invasive interventions such as the use of a rectal temperature probe.

Grip strength is often used as a test of muscle function. Traditionally, this was tested by measuring how long an animal could support their body weight using their forelimbs while hanging from a horizontal bar. We will use a less stressful grip strength meter which avoids suspending the animal above the ground.

In our ageing experiments, rather than measuring the full lifespan we will reduce the risk of

animals experiencing suffering associate with the end of life by measuring the healthy lifespan. To do this we will monitor animals aged over 78 weeks (having established that age related weight loss begins later for both sexes) by daily inspection and weekly weight measurements. Animals exhibiting loss of more than 10% body weight over one week, or other signs of frailty, including reduced mobility, abnormal respiration, solitary or circling behaviour, will be deemed to have reached the end of their healthy lifespan and will be humanely killed. Likewise, animals exhibiting signs of pain (hunching or piloerection), a visible or palpable (potentially cancerous) mass, or other visible defects will be humanely killed, unless they can be treated according to advice from the NVS. Results from our earlier experiments will inform work during this project such that optimal ages for analysis can be identified, including for animals representing old age but not dying. A similar monitoring scheme will be applied to new GA strains bred during this project, including those representing novel combinations of genetic alterations, with the aim of detecting unexpected effects from birth onwards.

Why can't you use animals that are less sentient?

Much of our work will involve studying development of tissues and organs at fetal mouse stages. Less sentient species cannot be used because the relevant genetic and lifestyle influences on growth and tissue proportions during development are likely to be specific to mammals. Certainly, the environment of the womb and the placental connection between mother and offspring is unique to mammals.

Imprinted genes, linked with the developmental programming of health and disease because they are unusually sensitive to adverse environmental conditions, are also found only in mammals.

Mice will also be studied at various post-natal stages because one objective is to determine how cell and molecular changes in early life can have a lasting effect on adult tissues and whole-body physiology. In most cases we can limit analyses of adult mice to a maximum of 36 weeks of age. Our previous work has shown that important changes in adipose deposition occur up to 36 weeks, including differences between males and females. The exception will be a minority of animals kept to study healthy ageing, typically for up to 24 months.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have adopted non-aversive handling methods and will use these routinely throughout the project. For procedures requiring fasting we have reduced the fasting period from overnight to a maximum of 8 hours during the daylight hours when feeding is already reduced. Blood sampling is carried out using a superficial vein, typically by making a small nick in the tail vein, where a drop of blood is sufficient (e.g. for a glucometer reading). For injections and blood withdrawal needles are used only once and with the smallest practicable gauge. When surgical procedures are used to derive novel GA strains, or to import or rederive from frozen existing strains, suitable analgesia will be administered and recovery carefully monitored.

To make the most of each animal in our physiology experiments we make multiple measurements of, for example, body weight, body composition (lean and fat mass estimates by NMR), blood pressure, grip strength, temperature, and glucose handling

(GTT and ITT). This allows us to acquire longitudinal data to reveal age-related effects. Post-mortem cellular and genetic analyses of multiple tissues can then be related to these physiological measures. To reduce the associated stress placed on animals within such studies the different tests are spread out to afford periods of respite. Where animals need to be housed individually, for example to measure food intake, these periods are kept to a maximum of 14 days.

In our ageing experiments we will study the healthy lifespan rather than the full lifespan, to avoid end of life harms as far as possible. To achieve this, aged animals will be carefully monitored by trained staff for signs of frailty, including declining body weight from 78 weeks of age. This time point has been established through our previous work, showing animals typically lose <1% of their body weight per week after this time point. Using a 10% body weight loss as an end point fewer than 7% of females and 4% of males died suddenly before they could be humanely killed, which was less than the 16% of spontaneous deaths among animals of younger ages. We will look to refine this monitoring system during this project. Group sizes will be designed to take account of the expected loss of animals at advanced time points, based on our previous survival data.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We have considerable experience in planning and conducting experiments involving mice and remain open to new ideas or improvements in practice. We will consult both the PREPARE and ARRIVE guidelines when planning experiments to ensure we use the most refined approaches. In addition, we will consult documents published by NC3Rs and the Laboratory Animal Science Association, along with relevant research publications evidencing refined approaches.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

In addition to reading the scientific literature we will regularly consult the NC3Rs website and read the NC3Rs newsletter, to which we subscribe. We will interact with the NC3Rs personnel when possible and attend events such as the Regional 3Rs symposia and 3Rs lectures organised by our AWERB committee. We will attend the regular establishment Animal User Committee meetings which is a forum for sharing best practice.

17. Maintenance and differentiation of mammalian stem cells

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Stem cells, Development, Homeostasis, Cell differentiation, Cell lineage

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Stem cells are cells with unlimited power to divide, yet remain able to become at least one specialised cell type. In this project, we aim to characterise stem cells and their derivatives during the development and maintenance of mammalian organs and tissues.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Stem cells can replicate to make exact copies of themselves (called ‘self-renewal’), and to make one or more different specialised cell type (called ‘differentiation’). The stem cells that are found in the very early embryo have the potential to make all of the different cell-type in the body. However once organs and tissues form, the stem cells that maintain them can usually make only cells of that organ or tissue. Stem cells are of great interest in fundamental research, because if we can understand how the choice to self-renew or differentiate is controlled we will understand the development and maintenance of complex

organisms, including mammals, much better. Stem cells are also a major focus for clinical applications, since they are responsible for repairing our organs and tissues. If we understand how they are controlled in the healthy body, we may be able to apply this knowledge to understand how to induce our own stem cells to better repair damaged organs and tissues, or alternatively we may be able to grown stem cells in the laboratory that can produce replacement cells for tissue/organ repair by transplantation. It is already possible to grow the stem cells from some organs/tissues in the laboratory. However, for many organs/tissues, our knowledge of how the stem cells arise and are regulated in the body is currently insufficient to allow the cells to be grown in the laboratory. We aim to determine the fundamental properties of stem cells and their descendant tissue-restricted stem, progenitor and terminally differentiated cells during mouse development and adulthood, and to test the same properties of mouse and human stem, progenitor and terminally differentiated cells that are grown in the laboratory. This will allow us to design and validate better ways of producing stem cells that can make different organs and tissues in cell culture, leading eventually to the development of new therapies. The work in this project will allow us to (1) better understand how stem cells and the differentiated cells they generate behave in intact animals, (2) provide a baseline to determine whether lab-grown stem and progenitor cells behave similarly to their equivalents in the body and (3) test whether cells derived and differentiated in the lab have potential for cell replacement therapies.

What outputs do you think you will see at the end of this project?

Our research will advance our knowledge of how the stem cells that build and maintain different organs and tissues are controlled in the body, so that they both maintain a pool of stem cells and replace dead or damaged cells as needed through the lifespan. This is fundamental research, so a primary output will be an **understanding of the fundamental biological processes that operate in mammals**. Our work will also test whether this knowledge can be used to improve methods for growing or making the stem cells from different organs and tissues in the laboratory, so **improved protocols for growing stem cells and their tissue derivatives will be another primary output**. In the medium term, at least some of our findings are expected to lead to **clinical approaches to curing disease and repairing injury**. The measurable outputs will therefore be: new knowledge, disseminated at scientific meetings and in peer-reviewed publications, and translationally-oriented pre-clinical studies that may lead to new therapeutic approaches.

Who or what will benefit from these outputs, and how?

We study stem cells when they first arise during embryo development and also when they are functioning to maintain and repair adult tissues, using a powerful shared approach which will lead to a holistic view of stem cell behaviour. This is expected to have impact at several levels. In the short/medium term, the beneficiaries of our research will principally be other basic and clinical researchers in Universities and in industry, who will gain new knowledge about different types of stem cell in the body. In the longer term, we expect some of the research to help develop new therapies.

For instance, we expect to identify ways of boosting stem cell activity in the body, and/or of generating specific types of cell in the lab from stem cells. This new knowledge is expected to contribute to new therapies that alleviate, cure or prevent a range of degenerative diseases, or improve the way that tissues and organs repair themselves after injury or damage, or age-related deterioration. Our shared approaches are relevant to

organoid biology, and to a range of applications including immune system recovery after radiation treatment and in aging (thymus progenitors); for treatment of motor neuron disease (neuromesodermal progenitors); and improved fertility treatments (primordial germ cells).

Overall, our work may therefore benefit the pharmaceutical industry, clinicians, and patients, as well as other researchers. As our work includes active development of methods for growing or generating specific stem cell-types in the laboratory, in the medium to long term, it is also expected to benefit animal welfare.

How will you look to maximise the outputs of this work?

We will publish our results in high-impact open access peer-reviewed journals; In the last project licence period (2018-2023), we published work performed under this licence in Nature, Developmental Cell, Cell Reports, Development and eLife. We have in the past published 'unsuccessful' approaches in journals like Molecular and Cellular Biology, which includes a section on hard-to-interpret or negative phenotypes, and will continue to explore this publication route where applicable.

Other journals like PLoSOne and Biology Open also provide a route towards publication of such 'negative' data. The benefit of publishing 'negative data' is that it reduces the likelihood that a hypothesis that has already been tested and proved wrong will be tested again in another lab (i.e. it should reduce duplication of research that is already known to be unfruitful). Preprint repositories allow dissemination of results without the positive selection bias of peer review for high-impact journals.

The advantage of publishing on 'pre-print servers' such as BioRxiv is that the work can be available in the public domain ahead of its publication in its final form, and this can help other researchers in the same field to develop their own research better. The outputs of the work will also be presented at scientific meetings.

Additionally, we typically make resources available to other researchers (e.g., data, animals, tissues) in accordance with Open Access guidelines (e.g. on request, at the point of publication, or at a defined time point after a project finishes).

Species and numbers of animals expected to be used.

- Mice: 18820

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We use mice in our experiments because this is the standard laboratory mammal used to study stem cell systems; its mammalian status means that physiologically it is relevant to human disease, and experience over many decades ensures optimal welfare of the animals. In addition, the mouse generation time is relatively short for a mammal, making it

more amenable for studying the development and maintenance of specific organs than longer-lived mammals. A large number of reagents for isolating and characterising cells from different organs and tissues is also available for mice (including a very large number of existing classical and genetically modified mouse lines that we can access), whereas the range of equivalent reagents available for other mammals is far more restricted. In our own laboratories we have already established genetically altered mouse lines (for instance mice carrying fluorescent reporter genes that mark the presence of a specific cell type). Since these strains have been kept in the lab for several years any adverse effects are well-characterised: the vast majority of genetic alterations that we use have no adverse effect on the animal.

We study embryonic, fetal and adult stages.

We study embryonic and fetal forms to determine how stem and progenitor cells arise in organs and tissues, and how they are regulated during these developmental processes - typically from the early- mid stages of gestation.

We use adult mice for breeding and maintenance, and to test the function of adult organs derived from these early stem/progenitor cells, e.g., in the thymus, we are investigating whether stem cell- or reprogrammed-cell generated thymus cells fully mimic the function of thymus cells generated in normal development, and it is only possible to test this in adult animals. Some thymus function tests are performed in aged mice. This is because the thymus undergoes a very early age-related degeneration that has a strong influence on deterioration of immune system function with age, and we are testing whether these effects can be reversed either by activating stem cells in the remnant thymus or by using transplantation of thymic organoids to boost thymus function in aged individuals.

Typically, what will be done to an animal used in your project?

The following are separate examples of the experience of typical mice on the project:
Wild type mice (fetal and adult) will allow us to examine stem cells obtained from normal tissues. For example, mice will be injected with substances, for instance with a substance that labels dividing cells, that can be used to identify whether specific cells are dividing rapidly or slowly.

Mice will be maintained that carry harmless genetic alterations (e.g. reporter genes or conditional modifications where gene function is altered only on treatment with a molecule that activates protein function). Such molecules may be administered either to adult animals carrying the required genetic modifications, or to pregnant females where the embryos carry the required genetic modifications.

Administration is typically by oral gavage (administration of the molecules in solution directly into the stomach, via a thin flexible tube), in the drinking water or in the food.

In most experiments such molecules are administered over a short period, but in some experiments administration of low concentrations over a period of a month is required (e.g. to test the effect of particular genes in the thymus function assay).

Mice which have impaired function of a particular organ such as the thymus, the organ required for T cell development (e.g. nude mice), will be maintained to use in experiments that attempt to restore this with transplanted organoids containing putative thymic

stem/progenitor cells. The organoids are transplanted under the kidney capsule, a standard surgical procedure. Organoids are 3-D structures that are produced in vitro and mimic the key functional, structural, and biological complexity of a specific organ. Organoids can currently be generated for a variety of different organs, including the thymus.
Aged mice whose thymus function is naturally minimal will be used to test whether we can reset thymic function either with stem-cell or reprogrammed cell-based transplant thymic organoids or by stimulation of the body's own cells (e.g. by inducing genes known to regulate differentiation of thymic stem cells during thymus development). In some of these experiments, the mice will receive a skin graft, a vaccine challenge or a tumour challenge (in which a defined number of tumour cells are implanted into the mouse, typically by injection at a defined site such as the flank). This allows us to test whether improved thymus function resulting from thymus organoid transplantation or from stimulation of thymus regeneration has resulted in stronger T cell-mediated responses than in aged-matched controls, and whether the T cell pool generated following thymus transplantation is fully functional but self-tolerant (i.e. does not contain auto-reactive T cells).

Mice are humanely killed at the experimental end points and tissues harvested for analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

The adverse effects of the genetic alterations are generally mild or sub-threshold. The large majority of our animals will not have any surgical procedure carried out on them: in most cases the endpoint is tissue collection with/without administration of substances, or the animals are used to maintain the breeding colony. All our mice, including those with impaired immune systems, are maintained in a specific pathogen-free environment and therefore mice with impaired immune systems are not adversely affected by this deficiency.

The surgical procedures used (for example for embryo implantation) are classed as moderate but do not by themselves cause lasting harm, since pain is managed before and after surgery under anaesthesia through analgesia, and animals are monitored post-surgery for signs of pain or distress, or problems with the wound such as infection. These are very rare but in these cases, with vet and NACWO advice, animals are either treated (for example with antibiotics), or humanely killed.

In thymus function tests that use transplantation of stem cells to restore thymic function, the procedure involves surgery and so is classed as moderate. However, the animals are kept under specific pathogen free conditions and therefore there are no expected long-term experimental harms due to these procedures. A rare consequence of the transplantation of cells can be tumour formation, especially if the cells injected have a pluripotent phenotype. This has not been observed in thymic experiments to date, but in case of signs of pain, distress or weight loss, with vet and NACWO advice, animals will be humanely killed to remain within the severity limits of the protocol.

In thymic function tests where functional protein expression is altered in aged mice in response to a protein inducer molecule, in order to induce thymus regeneration, the mice will initially be given the molecule orally and then in the drinking water or in the food for 1 month. These mice will be carefully monitored for weight loss, which can occur after oral

administration or due to reluctance to drink water containing the protein inducer molecule.

Mice that develop weight loss will be removed from the water containing the protein inducer molecule temporarily and fed mash to restore normal hydration. We are currently testing alternative methods for administering the protein inducer, including pipette dropping to replace gavage, and use of micropelleted mouse food to replace inducer containing water.

In the tumour challenge model tumour cells (from cultivated cell lines) are injected into flank skin. This procedure is also classed as moderate. Mice are monitored closely for tumour growth and are humanely killed if the agreed maximum tumour size is reached or if any other harmful effects are noted. This model is very well established and therefore the number of cells to be injected to achieve the required experimental endpoints, and the behaviour of the tumour cells following injection, is already known.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We expect a majority of animals (around 60%) will have sub-threshold effects, a smaller proportion mild (25%). A small proportion (approximately 15%) will have moderate effects.

What will happen to animals used in this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to use animals, specifically mice, in these experiments because the project aims to increase understanding of the very complex biological systems that regulate stem cell self-renewal and differentiation in vivo, during the organogenesis and maintenance of several different organs/tissues. A further aim is to use this information to develop methods for culturing these tissue-specific stem cells in vitro, such that they can still undergo controlled differentiation to generate their specific derivative cell types.

The use of mice is needed to allow characterisation of specific tissue stem cells under normal and experimental conditions, thereby generating data that increases fundamental understanding of how these cells are regulated in vivo. Use of mice is also required for functional testing of cell types harvested ex vivo; of stem cells and reprogrammed cells grown in the lab; and of organoids generated from these lab-grown cells. These functional tests cannot yet be performed in vitro, due to the complexity of the processes being assayed.

Which non-animal alternatives did you consider for use in this project?

Our research is on the properties and regulation of mammalian stem cells in specific organs and tissues during initial development in the embryo, and in the adult.

Because of this, our studies must be conducted in a mammalian species, and in mammalian cells grown in the laboratory.
We considered:
Non-protected species such as fruit flies or nematodes. We considered whether organ function was similar enough between these species and mammals to draw relevant conclusions.

In vitro cell culture approaches. We considered whether standard monolayer culture and 3-dimensional organoid approaches, including those we are currently developing in the lab, are at a stage where they can replace in vivo work.

Why were they not suitable?

We cannot use non-protected species such as fruit flies or nematodes, as they do not possess equivalent organs and tissues to those we are studying, although some information (e.g. on the regulation of some genes and some signalling pathways) obtained through studies in these species relates to fundamental mechanisms operating across many species and will be considered in our studies and advances in these species will be monitored in our ongoing studies.

Development of in vitro approaches (e.g. organoids) to the point at which they can replace in vivo experiments is very difficult, due to the complexity of the biological systems that must be mimicked, and has not yet been satisfactorily achieved for the cell types we are working with. We are committed to the 3Rs and will continue to develop these approaches in order to minimise and eventually replace the use of animals in our experiments.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of mice to be used are estimated based on the following principles of experimental design, in line with PREPARE and ARRIVE guidelines:
Choice of different experimental groups: All experiments will include control and test groups. In some experiments, we will use a design matrix to allow cross-wise comparison of different test conditions. For most of our experiments, the dosage (e.g. of substances) has already been determined, in experiments where this is not the case dosing levels will be determined in small pilot experiments.

Use of control groups: Control groups are used to account for the possible effects of all elements of the experimental model. Negative controls are used to determine if a difference between groups is caused by the intervention (e.g. wild-type animals vs genetically modified animals). Positive controls will be used if required to support the interpretation of negative results or determine if an expected effect is detectable. We do not expect to use sham surgical controls as the surgeries to be performed (e.g. thymic stem cell transplantation; skin grafting) are very well established and existing data indicate that such controls are not required.

Maximisation of data output from the animals: Data output from the animals will be ensured by experimental design that maximises the information that can be obtained from the experiment. If it is not possible to conduct some analyses immediately (e.g. for funding reasons), we will freeze the cells/tissues if possible under conditions that will allow the analyses to be conducted at a later date. We will also take steps to share any tissues not required for immediate analysis with other researchers working on similar questions; again, if appropriate, such tissues will be frozen until needed.

Determination and minimisation of likely variability: We will use standardised protocols to minimise variability. This will include standardisation of strain and substrain, sex, weight, and age of animals, both for in vivo studies and for studies using cells harvested ex vivo and used in in vivo experiments, and will also consider the effects of cage grouping for in vivo experiments. Where appropriate (e.g. in experiments assaying the effect of gain or loss of gene function in vivo), controls will be littermates.

How group sizes will be set: Group sizes will be set based on previous work, including previous pilot experiments. The group size will vary dependent on the size of the effect expected, and on the number of cells required for the endpoint analyses (e.g. for single cell RNAseq, single cell chromatin accessibility analysis, proteomics analysis etc). We will use the sample size calculator on the NC3Rs EDA website to assist with sample size calculations, and will also work with support statisticians as needed. For instance, a statistician helped us with calculations using typical variations from earlier experimentation using the tumour models we will use to assay the functionality of the T cell repertoire after thymus regeneration; this has set a group size of 12 for the experiments analysing T cell control of tumour growth.

Sample sizes for other experiments are also estimated from past experiments, and indicate group sizes varying from 3-9, depending on the experiment in question. We have used our annual return data to estimate the number of animals we will need to use for breeding.

Randomisation and blinding: We are aware of the benefits of randomisation and blinding.

This is often not possible, e.g. as only one researcher is available to work on a project.

However, we will implement randomisation and blinding where possible, following guidance in from NC3Rs, including use of the EDA.

Use of pilot studies: Pilot studies are used where appropriate to gather information prior to larger studies, to improve the quality and efficiency of the larger study, and to bring to light any deficiencies or problems with the design of proposed experiments. For most of the studies in this project, the pilot studies have already been performed, meaning this

information has already been gathered and used to inform experimental design. However, in cases where, for instance, a mouse model is being used for the first time, or is being used again after a long period of time, appropriate pilot studies will be performed ahead of larger scale experiments.

Comparisons between groups or experimental situations: Comparisons between groups will be made using statistical analysis where appropriate. Choice of analysis will be determined during the experimental design phase and will be informed by a statistician, the NC3Rs EDA tool, or equivalent, as appropriate.

Analysis of data to ensure the maximum efficiency of animal use.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We minimise the number of animals being used by using good experimental design approaches. Where appropriate, we will employ the NC3Rs' experimental design guidance and experimental design assistant (EDA) to plan our experimental design, practical steps and statistical analysis, including sample size calculations and appropriate statistical analysis methods. We will use the EDA diagram and report outputs to support experimental planning with animal users. Randomisation and blinding is not used in most of our experiments, but we are aware of the guidance and support on randomisation and blinding available through the EDA and will make use of this whenever possible and appropriate.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We plan breeding for maximum efficiency. Where possible, we share tissues between research groups. Where possible, we use computational modelling to inform experimental design. Additionally, where possible, we will share tissues between research groups (e.g. in experiments using aged mice tissues from control mice, that are not required as part of the main study, will be of interest to groups working on other organs and tissues; in experiments analysing loss or gain of gene function, if the loss of gain of function occurs in other tissues as well as the one under study, these tissues will be of interest to researchers working on those tissues).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use mice as the animal model of choice because mouse husbandry has a long history

of optimising animal welfare, and knowledge sharing of refinements in methodology is widespread. The genetic models we use generally have neutral (sub-threshold) or occasionally mild effects on the animal.

Where, rarely, an effect of a conditional mutation results in a moderate effect where there is no alternative for generating the data, animals will be moved to the moderate protocol.

However close monitoring and development of more refined practices will ensure that the vast majority of animals will not require this.

Several of the methods used are classed as moderate, including embryo transfer of chimeric recipient mice, skin graft, tissue potency assay and tumour challenge model. The classification of moderate is due to the use of surgical techniques as part of the protocol (embryo transfer of chimeric recipient mice, skin graft, tissue potency assay), or other moderate adverse effects of the protocol (e.g. tumour challenge model). These models are used as at present there is no alternative that can assay the equivalent biological functions in our experiments. The procedures minimise the pain, suffering distress or harm to the animals, for instance by the use of analgesia during procedures and by close monitoring of mice for weight loss and other indicators of discomfort following surgery.

Why can’t you use animals that are less sentient?

As a grouping of scientists interested in mammalian stem cells, with potential clinical applications in drug design and cell therapy, the mouse is the most-refined mammalian experimental model and is used for that reason. We use embryonic and fetal tissue for many of our experiments, but for characterisation and functional testing of the stem cells in which we are interested in adult organs and tissues there is no alternative to using adult mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We already have very stringent post-operative care and monitoring practices in place and will continue to implement these. We will introduce refinements to our protocols as they arise.

Ageing animals will be carefully monitored. The size of experimental groups in ageing experiments will be increased to accommodate for loss of animals and to avoid single housing due to animal losses due to old age. Animals will be monitored for adverse effects such as changes in weight, dermatitis, piloerection, paleness, changes in mobility, lumps, eye defects, abnormal respiration, or stools. If these are observed animals will be treated accordingly, and animals that develop severe effects will be humanely killed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow best practice guidance from NC3Rs, including the PREPARE and ARRIVE guidelines, to ensure our experiments are conducted in the most refined way possible.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We routinely monitor for new technology refinements to welfare e.g. through the NC3Rs newsletter. For example, we have recently changed from tail handling to the much more refined tunnel handling, which places much less stress on animals.

Training is available for all users to adopt this technique. We refer frequently to the NC3Rs website, which gives guidance on improved approaches to animal handling and to specific procedures. 3Rs advances related to the specific research area and techniques used in this project will be monitored through participation in field-related networks/groups that share this information.

18. Role of injury-repair programmes in the pathogenesis of epithelial tissues

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Development, Stem cells, Repair and Regeneration, Cancer, Ageing

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To determine how the molecular and cellular programmes that support the repair and regeneration of epithelial tissues following injury or inflammation become repurposed in ageing and during the transition to cancerous states.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Epithelial tissues have many important functions. As well as providing an essential barrier function, protecting the body from exposure to pathogens and toxins, epithelial tissues control the exchange of nutrients, enzymes, and waste products.

To maintain their function, cells lost through exhaustion or damage are constantly replenished by specialist cell populations known as stem cells. These stem cells are defined by (1) their capacity to duplicate through cell division and (2) give rise to the more

specialised daughter cell types that carry out the functions of the tissue, a process known as differentiation. To ensure that tissues are maintained over the long-term, these two processes must be perfectly balanced so that the size of the stem cell pool remains constant over time.

However, through the chance accumulation of genomic DNA mutations, these two processes can become imbalanced. In such cases, mutant cells and their daughter cells can expand in number at the expense of their normal (non-mutant) neighbours. This can lead to the development of patches or fields of mutant cells, a phase of pre-cancerous growth known as "field cancerization". This progressive expansion of mutant fields of cells increases the chance for further DNA mutations to occur, which can drive the development of cancers. These cancers can subsequently escape from the epithelial layer and invade into the underlying connective tissue, known as stroma. Here, cancer cells can spread from the tumour site to other tissues of the body in a process known as metastasis.

The aim of this project is to study the cellular and molecular mechanisms that control the fate of epithelial cells under normal (undisturbed) conditions and understand how these programmes become compromised (dysregulated) during the earliest stages of tumour development as well as in ageing.

Building on insights gathered from our own studies and the recent literature, we will challenge the hypothesis that the earliest stages of tumour development take advantage of the natural mechanisms that support epithelial repair following injury and inflammation. As well as providing fundamental insights into the basic biology of epithelial tissues, these studies may contribute to early detection methods by revealing new biochemical signatures (biomarkers) of disease and may lead to the development of new therapeutic intervention strategies that target the differentiation of tumour cell types.

What outputs do you think you will see at the end of this project?

With its emphasis on fundamental discovery-led research, the main output of this project will be the insights that are gained into the mechanisms that regulate the fate of epithelial stem cells and their daughter proliferative cells (known as progenitor cells) under normal and disturbed (perturbed) conditions. We will then use this knowledge to gain a deeper understanding of how these programmes become repurposed and corrupted during ageing and the transition to cancerous states. The emphasis of the project will be placed on epithelia of the mouse gastrointestinal tract (including the stomach, small intestine and colon), and the epithelia of the skin epidermis and oesophagus. However, to study whether and how mechanisms are conserved, our analyses will include comparative studies across other epithelial tissue types including branched epithelia such as the mouse salivary gland, pancreas and lung.

To trace the fate of mutant epithelial cells and their daughter cells – known as clones – we will make use of an established cell labelling technology based on genetically modified mouse models. Using specialised mouse models developed by our lab, we will quantify at single cell resolution the fate of mutant clones as well as their interaction with their non-mutant (wildtype) neighbours.

Together, these studies will:
expand our understanding of the cellular and molecular mechanisms that regulate stem and progenitor cell fate during the development and maintenance of epithelial tissues, and

in response to injury or inflammation;
quantify the impact of cancer-associated mutations - known as oncogenes - and oncogene combinations on the fate behaviour of mutant epithelial cells and their progenies, combining these statistical measures with an analysis of molecular, environmental and structural changes that take place during the earliest stages of cancer development – a process known as tumorigenesis;
quantify the impact of oncogenes and oncogene combinations on the dynamics of mutant epithelial cells and their progenies in response to injury or inflammation;
investigate whether and how natural injury-repair programmes become repurposed or corrupted in ageing and during the transition to cancerous states.

The impact of our research will be shared with the community through research publications, conference presentations, research seminars, and social media platforms. We will also be alert to opportunities to commercialise our findings, where there is potential for translation.

The genetic mouse lines developed under this licence will provide a valuable resource that can be generalized to other tissue types and oncogenic mutations, and used by other groups for the identification of novel biomarkers of disease, live-imaging of tissues, as well as investigating other cancer-related biological processes, such as metabolic changes, inflammation, and ageing.

Who or what will benefit from these outputs, and how?

By advancing our knowledge of the basic mechanisms that underlie the earliest stages of tumour development and ageing, the proposed activities will have an immediate impact on the research community through publications and the sharing of resources. More generally, all transgenic mouse lines generated through this project will be made available to the community, where they can be used to study epithelial tissues and readily adapted to other tissue types not considered in this project. In the long term, the product of this research has the potential to provide candidate molecular targets for use in the design of drug compounds in the context of regenerative medicine, as well as early cancer diagnostics and therapeutics.

How will you look to maximise the outputs of this work?

As a collaborative research group, we will remain alert to the opportunities to extend our work by sharing our biological materials (including chemical compounds and cells for 3D organ culture studies – the study of tissue and organ models in a dish), mouse lines, and the intellectual resource invested in our understanding of modelling-based analyses of clonal data. We will be ready to share our transgenic mouse models (existing and new), building collaborative partnerships with researchers whose interests extend to other epithelial tissue types, or with technological skills such as intravital imaging (the imaging of epithelial tissues in living animals), or spatial multiomics approaches (the quantification of gene expression patterns as well as non-genetic factors such as DNA conformation in the same individual cells) not currently available to our laboratory. Even within the scope of the current project, cell fate mapping studies of transgenic mouse models may provide data for other tissue types. In this case, we will readily share these materials with researchers who have expertise and interest in these areas.

Species and numbers of animals expected to be used.

- Mice: 29900

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

To understand the earliest steps in cancer development, it is important to determine the behaviour of tissues under normal (healthy) conditions. Previous studies by our lab and others show that, during the earliest stages of tumour development, epithelial cells often reacquire gene expression patterns of cell states found in tissues at late embryonic stages of development or soon after birth, a behaviour also observed in response to injury as well as in ageing. To gain insight into the pathways that drive the early stages of cancer development, it is important to combine organ culture-based models of human epithelial tissues with animal models, where the impact of environmental factors such as immune cells and the remodelling of stromal tissue can be studied.

To develop our research programme, we choose mouse as a model system. The mouse is the most appropriate animal model for our work since: (i) it is a mammal; (ii) its physiology is more extensively characterised than that of other mammalian model species; (iii) mice are amenable to genetic manipulation, enabling us to induce cancer causing DNA mutations at the level of single cells; and (iv) a large number of relevant genetically altered animal models are already available to the community.

To study the normal behaviour of epithelial cells during development, in adult and in ageing, we will make use of animals across all life stages, from the embryo, through postnatal stages of development, in adult, and on into ageing.

Typically, what will be done to an animal used in your project?

Administration of chemical agents by injection: The project licence lists several protocols, from breeding and maintenance to genetic lineage tracing, which include the administration of chemical agents to mice by intraperitoneal (i.e., into the body cavity) or subcutaneous (i.e., under the skin) injection. Such methods may be used to induce hormones, such as gonadotrophin, to stimulate the ovary to produce eggs during the breeding of animals, or they may involve the injection of drug- inducing agents such as tamoxifen or doxycycline to activate the expression of oncogenic mutations and/or fluorescent reporter genes in animals to enable the fate mapping of cells and their progenies.

Surgical procedures: In the breeding and maintenance programme, surgical procedures may be applied such as the surgical implantation of embryos into pseudo-pregnant mice or vasectomies. Vasectomized mice will then be paired with females to induce pseudo-pregnancy. Following surgical implantation of embryos into pseudo-pregnant mice, animals will be allowed to come to term.

Breeding and maintenance: Mice will be bred by conventional breeding methods and maintained in regulated facilities that operate under strict regulations laid down and

licenced by the Home Office. Animals kept for breeding will either be used for colony maintenance or transferred to experimental protocols associated with the licence.

Similarly, off-spring born from breeder animals will either be transferred to other protocols to be used for experimental purposes, or kept on the standard breeding protocols to maintain or expand the existing mouse line, or bred with a different strain to start a new mouse line. Mice with harmful phenotypes will not be kept as breeders beyond 20 weeks of age.

Animals will be maintained by methods appropriate to their genetic alteration, typically until they reach a maximum of 12 months of age. In the context of ageing studies, mice may be kept longer, up to a maximum of 24 months of age. Where possible, mice will be housed with their littermates and will not be singularly housed.

In either case, genetically altered animals are only expected to experience minor transient pain and discomfort.

Tissue biopsies: Tissue biopsies may be taken to determine genetic status by one of the following methods: ear punch (involving the collection of a small amount of tissue obtained from a hole punch through the ear using a specialised device), blood sampling, or hair sampling. Tissue biopsies will typically occur only once and will be taken from mice at a young age before being used for an experimental procedure.

Cell lineage tracing from embryonic time points: Typically, at the start of the experiment, pregnant mice from timed matings will be administered a gene-inducing or -deleting agent once by oral gavage, or by intraperitoneal injection if oral gavage turns out to be unviable as a reliable method to ensure consistent frequencies of cell labelling between animals. Oral gavage provides a method to administer a chemical compound orally using a small feeding tube with a bulb-like tip and attached to a syringe that is inserted gently into the oesophagus of the animals. Pregnant mice (including animals that have not been administered a gene-inducing or -deleting agent) may also be administered 1-2 times with a nucleotide analogue (BrdU or EdU) by oral gavage, or by intraperitoneal injection if oral gavage turns out to be unviable. The nucleotide analogues are chemicals that are incorporated into DNA during cell division and, when tissue samples are fixed, provide a method to detect the abundance and position of dividing cells at the time of administration. When the aim is to trace the fate of cells over an embryonic time window, both pregnant mice and embryos will be humanely killed and tissues collected for analysis. If cell fate mapping is carried out to time points after birth, mice will be delivered, and littermates will be humanely killed at the given timepoints. 1-2 days before killing, mice may be administered once with BrdU/EdU by oral gavage or by subcutaneous or intraperitoneal injection if oral gavage turns out to be unviable. In a given experiment, we expect to collect data from a range of induction times and over a range of time periods following cell labelling (known as chase times). For all lineage tracing studies, we will conduct pilot experiments to optimise the dose of gene-inducing or - deleting agents and to acquire preliminary data to validate and motivate a larger study.

Cell lineage tracing from postnatal and adult time points: Typically, at the start of the experiment, mice will be administered a gene-inducing or -deleting agent once by oral gavage, or by intraperitoneal injection if oral gavage turns out to be unviable as a reliable method to ensure consistent frequencies of cell induction. Mice will be humanely killed at the given timepoints. 1-2 days before killing, mice may be administered once with

BrdU/EdU by oral gavage or by subcutaneous or intraperitoneal injection if oral gavage turns out to be unviable. In a given experiment, we expect to collect data from a range of induction times and chase times. Once again, for all lineage tracing studies, we will conduct pilot experiments to optimise the dose of gene-inducing or -deleting agents and to acquire preliminary data to validate and motivate a larger study,

Chemical inhibitors and activators: The fate behaviour of epithelial cells is influenced by biochemical signals that can be altered in the context of mutant cells or with ageing. To study the impact of these biochemical signalling pathways, compounds have been developed that inhibit or activate their expression in tissue. To study the action of these chemical inhibitors and activators on the dynamics of normal (wildtype) and mutant clones, mice administered a gene-inducing or -deleting agent may be administered 1-3 weeks later with a signal-modulating chemical compound by oral gavage, or by another route depending on the specific guidelines of drug administration, once per day for 3-10 (average 5) consecutive days. The animal will humanely killed on the day following the final administration of the signal-modulating chemical compound.

Cell lineage tracing under perturbed conditions: Typically, at the start of the experiment, an adult animal will be weighed and administered a gene-inducing or -deleting agent once by oral gavage, or by intraperitoneal injection if oral gavage turns out to be unviable. After 1-4 weeks, the animal will then be subject to a mild injury of the epithelium, where the method of injury depends on the tissue of interest; either the gastrointestinal (GI) tract or skin epidermis. In the case of the GI tract, agents will typically be administered orally, or by another relevant route depending on the specific guidelines of drug administration, once per day for 2-5 (average 3) consecutive days. In the skin, either a shallow epidermal wound will be created, or a chemical compound will be applied topically with a maximum of 4 applications per day for treatments that are <10 days, and a maximum of 2 applications per day for treatments over 10 days, with a maximum of 20 days treatment. Finally, the animal will be humanely killed typically after 1-8 weeks following the final administration of the chemical compound or wound.

In the event of unexpected post-wounding complications at the epidermal wound site in the skin, animals will be humanely killed unless they respond to minor medical interventions within 24 hours of the injury. In the event that irritation does not resolve within 14 days, or the irritated region exceeds beyond 1 cm in circumference around the wounded area for more than 7 days, animals will be humanely killed. In the unlikely event of a visible non-healing wound, animals will be humanely killed.
Humane killing: At the end of a regulated procedure, mice will be humanely killed.

Mice may be humanely killed even if no regulated procedures have been performed either so that tissues can be collected or because animals have reached the end of their breeding life.

What are the expected impacts and/or adverse effects for the animals during your project?

Administration of chemical agents by injection: Mice that are administered chemical agents will experience mild transient pain caused by the injection.

Surgical procedures: Where surgical procedures are applied, some animals (<5%) may experience post-operative complications such as infection, wound dehiscence (bursting of

the wound), surgical or anaesthetic complications, or poor recovery, pain, or distress. In the event of post-operative complications, animals will be humanely killed unless, in the opinion of a veterinary surgeon, such complications can be remedied promptly and successfully using no more than minor interventions.

Minimally inflamed wounds without obvious infection may be re-closed on one occasion within 48 hours of the initial surgery. In the event of recurrence, the advice of the veterinary surgeon will be followed.

Cell lineage tracing from embryonic time points: The administration of a gene-inducing or -deleting agent to pregnant mice is necessary to induce the expression of the targeted transgene such as a fluorescent reporter gene. Although for our current genetically altered mouse lines the optimal dose concentrations have been established, new mouse lines might tolerate a different concentration, which might result in late foetal abortions and delayed delivery (estimated at <5%).

Cell lineage tracing of mutant clones: To achieve our project objectives, emphasis will be placed on the early stages of tumour initiation. The models we use are, by their nature, tumour prone. We anticipate that a minority of mice (estimated at <15%) in which oncogenes are induced may experience moderate adverse effects caused by the development of early tumour growth. Tumours may grow internally or as visible lesions on the skin. We consider the following signs associated with tumour growth to be moderate: piloerection (like goosebumps), hunched posture, and/or subdued behaviour, underactivity, diarrhoea, jaundice, altered respiratory rate, abdominal distension or neurological signs such as gait disturbance. Individuals displaying the clinical signs listed above will be humanely killed unless signs are resolved within 24 hours. If any animal shows body weight loss of more than 10%, the animal will be monitored daily until the animal shows signs of weight recovery.

Any animal that loses 15% of its body weight or more for more than 48 hours will be humanely killed. For a couple of mouse lines, genetic altered animals will spontaneously develop tumours known as premalignant adenomas as they age, with tumour development expected to occur between 25-40 weeks of age.

Cell lineage tracing under injury conditions: Animals are expected to make a rapid recovery following injury of the GI tract or skin epidermis. However, for the injury models of the GI tract, drug treatments may result in transient weight loss. If any animal shows body weight loss more than 10%, the animal will be monitored daily until the animal shows signs of weight recovery. Any animal that loses 15% of its body weight or more for more than 48 hours will be humanely killed. The maximum weight loss endpoint for all animals is 18% weight loss, if any animal reaches this limit it will be immediately killed.

Appropriate weight comparisons will be made by monitoring the weight of the individual before administration of the agent. For the skin epidermis, wounded areas may show transient swelling, which is typically resolved in 48 hours. The wound healing is naturally complete within approximately 14 days without any intervention.

It is expected that the use of hair removal cream or razors may cause skin abrasion.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Sub-Threshold: 48% (14400 animals)
Mild: 39% (11690 animals)
Moderate: 13% (3810 animals)

What will happen to animals used in this project?

- Killed
- Kept alive at the establishment for non-regulated purposes or possible reuse

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In the current licence, our aim is to achieve a comprehensive understanding of the mechanisms that regulate cell fate during the development and maintenance of epithelial tissues and how these programmes become altered in response to injury, ageing, or following the acquisition of cancer- causing mutations. While insights can be gained from the study of 3D organ cultures, the knowledge obtained from the investigation of the mouse is incomparable. Only animal models allow the reaction of stromal cells, immune cells and other components of the tumour microenvironment to be studied following the activation of cancer-causing mutations.

To achieve our aims, we have generated optimized genetically altered mouse models tailored to the study of the earliest events in cancer development. Using this approach, we will be able to quantify how mutations impact on the fate of epithelial cells, targeting both tumour cells as well as the reaction of surrounding environment.

Which non-animal alternatives did you consider for use in this project?

Ultimately, our aim is to translate our findings to the understanding of human tissues, particularly in the context of ageing phenomena and disease. Therefore, our animal research programme proceeds in concert with the study of both 2D and 3D cell culture models using primary tissue from both mouse and human.

Specifically, in the context of the gastrointestinal tract, our lab has experience in the use of cell culture systems using established protocols based on 3D organoid models (stomach, small intestine, and colon) and 2D air-liquid interface culture systems (colon), with plans to develop more advanced 3D organ cultures based on new state-of-the-art designs. In the former, 3D tissue-like structures can be grown under culture conditions from individual cells in a process that mimics the organ context. In the latter, epithelial cells can be maintained as two-dimensional single cell layers called ‘monolayers’ while maintaining the molecular identity and fate behaviour of the true tissue environment. Together, these models provide a platform both to screen the effect of chemical compounds that activate of

inhibit specific biological pathways, providing an opportunity to functionally validate targets from our animal studies. At the same time, they enable us to translate our findings to the study of human tissue.

Alongside these models, our lab also has experience in the use of models based on culturing tissue sections – known as explants – from the developing mouse colon, which has enabled us to circumvent technical challenges with our animal models, with the benefit of providing ready access to live-imaging data.

In the context of the skin epidermis and oesophagus, our lab has experience in the development of 3D organ culture systems based on a design developed and refined by our colleagues. By seeding small explant samples of mouse and human epithelial tissue, 3D organ cultures can be derived that can be maintained over the long term (from months to years) and which mimic the architecture and organization of the real tissue. By using our genetically modified mouse lines, we plan to trace the dynamics of wildtype and mutant clones in culture under normal and disturbed (perturbed) conditions, collecting clonal data in time-lapse. This “epithelioid” technology has been established for a broad range of epithelial cell types, creating a horizon to expand our analyses to other tissue contexts.

In the context of branching tissues such as the salivary gland and the pancreas, the lab has experience in the use of 3D organoid cultures of epithelial tissue as well as explant cultures using both mouse and human tissue. Further refinements of these models are expected during the course of the new project licence. These models will enable us to develop functional validation assays by exploring the impact of chemical compounds as well as addressing whether and how mechanisms of cell fate are conserved between mouse and human.

Together, these methods will significantly reduce the number of animals required to conclude our studies. Finally, we will remain alert to any advances that will enable the replacement of animals.

Why were they not suitable?

While insights can be gained from the study of 3D organ culture systems and organoid cultures, to study the fate behaviour of stem and progenitor cells during normal development and in response to injury, ageing and oncogenic mutations, the knowledge obtained from the investigation of the mouse is incomparable. Only animal models allow the reaction of stromal cells, immune cells and other components of the tumour microenvironment to be studied following the activation of oncogenic mutations in epithelial cells.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

In animal studies, it is often important to make use of blind or randomised trials in which animals subject to an experimental procedure are mixed with animals in an untreated (control) group in a randomised manner that is unknown to the investigator during the course of the experiment. Such an approach ensures objectivity in the analyses of results and avoids the potential for biases. However, for our animal experiments, it is usually not possible to blind or randomise mice as we are not, for example, comparing two treatments but are instead looking at normal or perturbed biological processes and regulatory programmes among cohorts of genetically altered (GA) mice. However, we will use blinding in the analysis of tissues collected from different groups of mice to control for scoring bias. This requires measurement of the given process, for instance clone size, cell type composition, or the rates of cell divisions, between GA and control mice, and the magnitude of the difference will determine the cohort size (number of mice) needed to achieve statistical significance. A refined estimation of cohort size will be obtained based on pilot studies.

When designing the experiments, we will perform statistical analysis to ensure that we use the minimum number of mice per experiment that will be informative and deliver definitive results that can be quantified based on their statistical significance – a formal measure of the probability that erroneous conclusions could be drawn because of chance outcomes due, for example, to small data sizes. A strength of the proposed approach is its reliance on mathematical modelling-based approaches that allows the fate behaviour of cells – their cell division rates, renewal (i.e., symmetrical cell division) probabilities, and the relative frequencies with which they give rise to more specialised daughter cell types – to be inferred from the statistical analysis of clonal outcomes. Within this approach, we use the statistical analysis of clone sizes and cell type composition over a given time course, to determine quantitative models of cell behaviour.

Although the projected mouse numbers for any given experiment will depend on the complexity of the underlying cellular dynamics in the appropriate condition, with some 100 clones or more per tissue sample per condition, the experience of our lab and others shows that statistical significance can be achieved from a minimal number of mice (3-5 mice per group). Depending on the context and the complexity of cell fate behaviour (such as the multiplicity of distinct cell states), to trace the fate behaviour of cells in wildtype mice and mice harbouring oncogenic mutations, we anticipate quantifying some 3-7 time points per mouse line. Indeed, the ability to abstract precise quantitative information from a minimal number of mice based on the statistical analysis of clonal outputs is a signature of our approach and a strength of the project licence. It also enables us to make a reasoned and meaningful estimate of the total numbers of animals required to conduct our work, reducing the number of animals required to a minimum.

Although the induction frequency may vary between epithelial tissue types, some of our mouse models allow for lineage tracing across multiple tissues from the same animal at the same time, leveraging the value of the approach and effectively reducing the number of animals.

The number of mice needed for isolation of organoid generation will depend on whether organoids are to be formed from dissociated tissue or from FACS (fluorescence activated cell sorting) sorted cells. In general, each organoid isolation would require up to 10 animals, and 3 separate isolations per genotype would be needed to ensure statistical reproducibility.

Note that, although we have been guided in the past using the NC3Rs Experimental Design tool, the analytical methods proposed here mirror those used by our lab in previous studies and we think have been optimised for our purposes.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

I have a training and research background in statistical physics and am conversant with the necessary mathematical expertise that will inform the experimental design.

At the same time, I will be ready to receive and respond positively to insight and advice from local statisticians and the wider research community.

In addition, we have optimized the following points on our previous licence:
Breeding optimization: We have optimized our breeding strategy to increase the chances of getting correct genotypes. This breeding strategy will also be beneficial for the introduction of new mouse lines that therefore would require fewer crosses to obtain the correct combination on alleles.

Tamoxifen optimization: we have titrated the correct concentration of tamoxifen for multiple routes (oral gavage, i.p., subcutaneous) and life stages (pregnant, neonates and adults). Therefore we do not have to test a very wide range of concentrations when testing new mouse models.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

To reduce the number of breeding pairs, the mice will whenever possible be kept as homozygotes (having inherited the same genomic sequence of a given gene from each biological parent). However, we note that there will be a need to breed from heterozygotes (with only one copy of a mutated sequence) too, to avoid the risk of mutation selection.

In addition, in protocols that include a timed mating step, a missed plug could lead to the birth of litters that have not undergone any regulated procedures. As these pups can still be used for breeding purposes, we have now implemented an optional step that allows the transfer of these animals back to the breeding protocol, thereby reducing unnecessary animal wastage.

As appropriate, we will undertake pilot experiments, particularly with cancer models, taking advice and training from our experienced collaborators, to ensure that we can safely monitor tumour progression to stay within the moderate limit.Chemical compounds (activators or inhibitors of biological pathways) will be pre-screened in pilot experiments to obtain an indication of the dose that is likely to be effective. As we will use only previously validated compounds, the starting dose will be set at the minimum to have an effect according to literature.

Tissues other than those that named on the licence might be affected by the candidate genetic perturbations since some mouse models allow activation of oncogenes across all organs. To maximise the information from a single animal, we will collect samples from respective organs and share these with other scientists, mitigating the need for further

breeding and experimentation.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.
To develop the proposed programme of work, we will make use of genetically modified mouse models tailored to the study of tumour development. The mouse is the most appropriate animal model for the proposed studies since: (i) it is a mammal; (ii) its physiology is more extensively characterised than that of other mammalian model species; (iii) mice are amenable to genetic alteration; and (iv) a large number of relevant transgenic and knock-out lines are already available. The experiments detailed here will involve creating and analysing transgenic mice and are classified as mild to moderate with respect to potential discomfort, stress, or suffering.

Mice will receive tamoxifen, doxycycline or Diptheria toxin (DT) administration to activate fluorescent reporter genes, oncogenes, ablate targeted cell populations, or induce a senescent phenotype. In our previous licence, we optimized the delivery and timing of tamoxifen administration to pregnant females by oral gavage, which reduced the handling stress to the females and increased the survival of pups during the treatment.

To study the dynamics of tissue regeneration in the colon, we will use a chemical treatment (using dextran sodium sulfate – DSS) at low dose to create a small, ulcerated region that typically repairs over several weeks. DSS is a well-studied method used by the community to model ulcerative colitis in animal models. In studies on our previous licence, we have mapped in full the response of animals to DSS treatment. Based on these findings, we have optimised the precise dosing and duration of DSS treatment for a aims and can predict the response to this epithelial injury over time. This means that we can now apply diet gel and heat pads before adverse effects (weight loss) are expected to occur.

To study the dynamics of tissue regeneration in the skin epidermis, we will use of inflammatory agents that have been tried and tested by colleagues in the research community or, alternatively, we will induce small superficial epidermal wounds to study wound healing.

By using transgenic animal models, mutations will be typically induced at very low (clonal) density. As well as enabling quantitative information to be inferred on the dynamics of cell fate under normal and mutant conditions, such an approach ameliorates the potential adverse impact of oncogenes on the animal. By focussing on the earliest stages of tumour development, the majority of animals experience minimal pain, suffering or distress. Moreover, our proposed injury models are chosen to ensure that the adverse effects on the animals are transient and cause minimal harm and distress.

Why can't you use animals that are less sentient?

While studies of less sentient model organisms (such as, e.g., flies, worms, frogs, and fish) have provided key insights into the mechanisms of epithelial stem and progenitor cell fate, the architectures of the tissues and the immune cell repertoire are too dissimilar to draw robust comparisons with human.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice will be maintained in a specific-pathogen free environment in individually ventilated cages. Mice will receive tamoxifen, doxycycline and DT administration, which normally do not harm mice. These agents will be administrated mostly via intraperitoneal injection, the pain of which is considered to be mild. In relation to the use of Tamoxifen, we will use our internal guidance to support our studies and animals.

DSS is one of the most well-characterized agents in rodent models of colon epithelial cell regeneration. For each mouse strain, we will refine our model by determining the dose that is sufficient to cause reproducible partial epithelial loss with minimal clinical signs. For example, the general dose of DSS administration used in the literature is 3% DSS solution in drinking water. Based on our previous studies, we will set this dose as the maximum, while aiming to use a lower dose, if suitable, to address our scientific questions.

For the gene knock-out studies, since we will use mouse models where mutations are only induced by the administration of drug-inducing compounds, mice should not display a phenotype before induction. To avoid unexpected pain and suffering, animals will be first bred and analysed as heterozygous animals. We will only use well-established reagents and protocols to induce expression or deletion of candidate gene(s). Taken together, the overall harm to mice that can be caused by performing the experimental plan will be minimal.

Finally, to minimise distress, we will limit the use of single housing. We will ensure only single needle use. We will ensure that animals are handled using a tunnel or cup. Dietary supplements may be used to reduce the risk of, or ameliorate, weight loss or treatment for skin conditions following wounding.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

To follow best practice, we will take advantage of several best practice guidelines including the ARRIVE guidelines that provide guidance on ensuring that the results of animal experiments can be reported in a manner that is scientifically rigorous and reproducible. To ensure that our experiments are compliant with the principles of NC3Rs, we take advantage of NORECOPA's PREPARE guidelines: PREPARE (norecopa.no), the NC3Rs, the ATLA (Alternatives to Laboratory Animals) and the LASA Guidelines. We will also remain alert to any advances that will enable the replacement of animals.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly check information on NC3Rs website, stay alert to advice from our local AWERB committee, and we have signed up to the NC3Rs newsletter. We will also engage frequently with our Named Veterinary Surgeon and our Named Animal Care and Welfare Office, as well as our Named Information Officer, our Named Competence and Training Officer, as well as our internal licencing support team to learn about the latest advances in the 3Rs based on their personal experience.

Last, but not least, the overall strategic aims and experimental designs in the current licence have an overlap with those of our collaborators and colleagues. This network provides a forum to share best practice and refine our experimental design to minimise suffering and reduce animal numbers.

19. Increasing our Understanding of the Pathogenesis of Pulmonary Hypertension and Developing New Therapies

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Cardiovascular, Pulmonary Hypertension, Lung Hypoxia, Oxidative Stress, Therapy

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant
Rats	juvenile, adult, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This research programme will provide new insight into the disease characterised by high blood pressure in the lung, called pulmonary hypertension, which has no cure.

We will investigate and characterise everyday communication between biological molecules within the healthy cell, particularly the role of proteins that can 'sense' the level of cell metabolites (i.e., caused by oxidative stress) in the lung vessels and help maintain the healthy vessels' state. We will use pre-clinical disease models and cutting-edge assessment, including various non-invasive longitudinal imaging modalities, to discover and test novel protein modifications and signalling pathways, assess new therapies that prevent and/or reverse the injury by interacting with these pathways and facilitate the

development of novel prognostic and/or diagnostic imaging tools for this detrimental disease, with the ultimate aim on clinical translation in the future.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

By understanding these processes better, novel therapies may be developed for patients with acute or chronic pulmonary vascular disease.

What outputs do you think you will see at the end of this project?

By the end of this research programme, new genetically modified mouse lines will be created, new therapies that prevent and/or reverse lung disease will be tested, and new imaging technologies will be validated. New knowledge and scientific information about disease mechanisms will be generated, and results that are not commercially sensitive will be submitted for publication in open-access peer- reviewed scientific journals. New results will prompt new grant submissions to internal and external granting bodies, as well as new internal or external scientific collaborations.

Who or what will benefit from these outputs, and how?

Our previous studies were performed in lung vascular cells, vessels, or rodent models of lung vasculature disease - such as high blood pressure and structural alterations in the lung vessels, also known as pulmonary hypertension (PH). In the past, we identified potential novel therapeutic targets for this rare but detrimental and incurable disease. The studies proposed in the new programme will further substantially increase and deepen our understanding of the cellular, molecular and systemic changes that ensue and cause the development of acute and/or chronic lung vascular disease with its ultimate progression to heart failure. The work carried out under this new project licence and new experimental data will be of benefit to other scientists in the field and the broader scientific community, as they will be made available in the form of conference oral or poster presentations in the first instance and as scientific publications later. Newly created genetically altered rodents will be made available for the broader scientific community on a collaborative basis after original data is published. Drug discovery studies will be important for the translational benefit of the proposed work. Finally, in the long term, this work could lead to new clinical trials, offer real human health benefits for existing and new patients, and improve clinical practices by implementing new and novel imaging technologies.

How will you look to maximise the outputs of this work?

During the last few years, we established new collaborations with experts in the field. Our research connections are broad and international, and these ties will ensure wider recognition and reproducibility of our work and maximise the potential outputs arising from each study. With this, we routinely disseminate our work as new open-access submissions for publication in peer-reviewed scientific journals and present our work at national and international conferences regularly.

Species and numbers of animals expected to be used

- Mice: 4000
- Rats: 1800

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The potential translation of preclinical work and applications in human patients crucially depends on whole-organism studies in which the whole-body physiology approach can be integrated into one setting. Rodents, particularly mice and rats employed in this work programme, most closely resemble the progression of the human disease model overall. We will primarily use young or adult mice or rats in pulmonary hypertension disease models, as the disease can affect young people and people of middle or older age. We will employ male and female animals whenever possible because this disease can affect both men and women.

Typically, what will be done to an animal used in your project?

Typically, an animal will undergo a breeding step (mice only), and once in adult age, it may be implanted with a surgically implantable probe to measure blood pressure. Rats or mice may be subjected to environmental changes such as low oxygen (also called normobaric hypoxia) for a fewweeks using specially designed chambers to induce sustained high blood pressure in vessels that connect the heart and the lung.

Some animals may also be treated with agents that make vessel cells grow abnormally and cause significant structural changes in lung vessels, which are also observed in human disease. This may be done in combination with a low-oxygen environment. Some animals, particularly rats, may be treated with the plant alkaloid monocrotaline, which causes damage to the inner wall of lung vessels, leading to structural changes and high blood pressure. The disease severity in all animals will be assessed using non-surgical and non-invasive body imaging modalities, including but not limited to echocardiography, computer tomography or MRI under general anaesthesia to obtain a health status. Some animals will be treated with drugs with disease-modifying capabilities before or during being subjected to a disease model of high lung blood pressure (i.e., disease prevention).

In some cases, to better mimic a real-life human scenario, a potentially therapeutic drug may be given to animals after the disease has been developed or fully established (i.e., to cause the reversal of the disease). During the course of the disease and post-disease establishment (up to 9-10 weeks altogether at the longest), the animal will be non-invasively assessed several times (with or without administration of contrast agent). In some animals, pressure measurements in the heart will be taken under general anaesthesia before being humanely culled and tissues collected for further analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

During breeding, established or new genetically modified mice will not be significantly affected regarding animals' general health and well-being (i.e., phenotype). However, some existing or new genetically altered mouse models may have specific genetic traits that may alter their response to disease progression but are not expected to show any clinical signs or adverse effects during breeding and maintenance, although they may show a worsened/ improved phenotype when subject to lung injury. Modelling a disease of high blood pressure and structural changes in the lungs by exposure to a chronic low-oxygen environment is usually well tolerated by mice and rats. This observation is supported by our nearly decade of experience and the experience of other people running similar studies in labs worldwide. Animals chronically maintained in a hypoxic environment may lose approximately 10% of their body weight within the first 1-2 days of hypoxic exposure and demonstrate reduced activity and hyperventilation. However, acclimatisation occurs (e.g., increased levels of red blood cells and potentiated haemoglobin synthesis), and animals return to normal activity, eating behaviour, and weight gain within a few days. Although very rare, some animals may die in a hypoxic chamber due to the adverse effects, which may include alterations in blood pressure and/or heart failure. At the same time, the most significant expected impact will be the development of right heart enlargement, which we will use as a readout of disease severity in both rats and mice. However, it will be more pronounced in rats. When injected with therapeutic or imaging agents, local tissue infection may rarely be shown as mildly swollen for no more than a few hours. Occasionally, during non-invasive imaging, a quick and transient drop in body temperature can occur, but we will monitor this by employing specifically designed temperature-controlling pads. If surgical implantation of a blood pressure probe is performed, very rare adverse effects after the surgery may include wound infection or the late development of heart failure (after a few days) due to unique, rare anatomical traits in some animals.

The signs for the latter may be manifested as increased respiratory rates, reduced activity, piloerection and hunched posture. The typical duration animals will be maintained for 6-8 months but no longer than 12 months for breeding/maintenance.

Any animal on an experimental protocol will not be kept for longer than 15 months of age and no longer than ten weeks following lung injury, typically to see the recovery from the disease.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice will be expected to be under mild (more than 60%) or moderate (less than 40%) severity. Rats are expected to be under mild (around 50%) or moderate (around 50%) severity.

What will happen to animals used in this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In this research programme, we aim to understand the mechanisms better and test new experimental treatments of a complex multi-factorial and incurable disease such as pulmonary hypertension. It starts with high blood pressure and progressive structural changes in the lung vessels and ultimately leads to heart failure and patient death. Such a fatal outcome of the disease necessitates observing chronic physiological changes within the whole organism during various disease development stages.

Furthermore, pulmonary hypertension affects more than one vital organ and in human patients in particular, the disease-related changes develop over several weeks to months and years. While we can model this disease for several weeks in rodents, we cannot 'mimic' it successfully in an experimental test tube or a Petri dish using cell monolayers, organoids or engineered vascular tissues, for example, as this will fail to provide helpful information related to multi-organ effects and knock-on effects from one vital organ to another. Such information regarding how therapies may be later tested and used in human patients is crucial. Therefore, animal research and models are required to understand the disease development and its effects on body health and to test any potential therapeutic strategies in the intact animal. We will only test treatments in animals for which there is a sound scientific basis based on our findings from in vitro experiments in cells or tissues, perfused animal organs and human post-mortem tissues obtained during lung transplantation from patients with this disease or healthy donors that die of different causes.

Which non-animal alternatives did you consider for use in this project?

Where possible, we use cell models/human tissue to identify potential pathways that may be important in disease pathogenesis and to test new therapeutic agents before embarking on animal studies. We routinely use human and rodent cells cultured in laboratory dishes to provide initial important information before embarking on animal experiments. We considered using vascular cell organoids, engineered tissues and co-culture of more than one vascular cell type, or human vascular cells derived from induced pluripotent stem cells (iPSCs), but they are suboptimal, as explained below. Due to our ongoing collaborations, we are lucky to perform complementary studies of human tissues or primary vascular cells isolated from pulmonary hypertension patients who underwent lung transplantation and donated their lung tissues for research. Using computational models is not currently possible, although our findings may guide future programs. In the future, artificially constructed data networks (e.g., network medicine) might help to predict the likely course of a disease and its response to treatment.

We will cross-check and further support our preclinical data with targeted gene-based approaches like mining exome sequencing data in the UK BioBank.

Why were they not suitable?

While we will still consider developing cutting-edge approaches related to mimicking a disease in a dish in the future, such as vascular cell organoids, engineered vascular tissues and/or co-culture of more than one vascular cell type, or human vascular cells derived from induced pluripotent stem cells, as well as blood outgrown endothelial cells, none of these techniques can fully replicate the course of the disease in a living system and fail to provide valuable and crucial information related to multi-organ effects and knock-on effects from one vital organ to another. This will affect translational and drug-discovery studies and how new disease progression assessments or therapies may be subsequently used in humans. Human tissues are not ideal as such tissues are difficult to obtain. Available specimens are often unsuitable for research purposes in terms of quality and usually come from uncontrolled populations.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers are robustly based on our 15+ years of expertise, significant training in physiological and pharmacological research and long-term care of rodents (both rats and mice) in various models of diseases, including the one proposed in this project.

The number of mice is significantly higher than rats as it includes mouse breeding protocol, which typically requires at least 10-15 breeding pairs per year, each of which would breed at least 5-6 times, producing on average 6-8 offspring pups per breeding cycle. This will serve us well in performing the research proposed here at a minimal animal number cost. Our careful use of selected targets will limit the number of animals used in the pre-clinical stages of this research programme.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All experiments will be carefully planned well in advance and discussed by the licence holder, deputy, group members, and other members of the Departmental community and collaborators to ensure that the experiments are scientifically valid and no unnecessary work is performed. As described above, preliminary work using cells and previously harvested tissues will eliminate unsuitable candidates, which will not progress to new whole-body studies, thus reducing the number of animals used in the future. Furthermore, testing new therapies will be initiated slowly, with pilot studies using a small number (n=3) of animals; pilot experiments will be performed on one sex only before validating important findings on the other sex. An appropriate statistical power analysis will be used to ensure that the minimum number of animals is compatible with proving our scientific hypotheses. Various statistical programmes could be employed (e.g., GraphPad or the commonly used NC3Rs The Experimental Design Assistant: https://www.nc3rs.org.uk/our-portfolio/experimental-design-assistant- eda). We will also consult the medical statistics specialists available within the Department to advise on experimental design. Finally, by

combining tissue from one animal, we can study the consequences of more than one pathology affecting more than one organ (e.g., heart and lung). This reduces the number of animals required by half.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

All efforts will be made to minimise and restrict (reduce) animal numbers. Measuring blood pressure in animals with surgically implantable devices and employing state-of-the-art non-surgical and non- invasive imaging techniques (e.g., ultrasound, photoacoustic ultrasound, PET or MRI) permit direct data comparison before, during and after the treatment or intervention.

Repeated blood pressure and non-invasive measurements permit animals to serve as their own controls. While remote telemetry is a gold standard for blood-pressure measurements in rodents, non- invasive ultrasound is a gold standard for the assessment of cardiac function. Both approaches are highly sensitive and robust techniques, providing several important physiological parameters from one single assessment, and where animals can serve as their own controls.

Where possible, multimodal imaging will be used to study two or more imaging agents simultaneously in the same animal, or two imaging modalities will be used at the same time, e.g. we can perform heart imaging with photoacoustic ultrasound to image anatomical and functional changes and oxygen saturation in the same animal simultaneously.

The introduction of non-surgical, non-invasive imaging techniques (e.g. echocardiography) and measurements similar to those used in patients means that the number of groups of animals can be effectively reduced because we can track the progression within an individual animal and thus maximise the data collected from each animal used.

We also routinely share frozen tissues or proteins/DNA isolated from animal tissues with other groups and will continue to do so, further optimising and reducing the number of animals used in research.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Historically, early studies into short-term physiological effects of low-oxygen environment on lung vasculature were performed in larger animals, like cats or dogs, and also large cattle, particularly for studying how prolonged low-oxygen environment exposure causes

structural changes in vessels.

However, miniaturisation has recently allowed us to use smaller animals, particularly mice and rats. The mouse has become the preferred animal of choice for many studies due to its adaptability. It is cost-effective to house within animal facilities in large numbers, has a short gestation period, and breeds readily and repeatedly, just as it does in the wild. This makes it the species of choice for manipulating its genome to test the role and causal effect of specific genes, proteins, or protein modifications.

Animal models for rats and mice under the proposed work programme are the best characterised and most widely used. They do not require complicated surgeries (e.g., systemic artery-pulmonary artery shunting) or lead to severe suffering or death and generally are considered fully reversible within a few months. The availability of genetically modified mice allows the determination of the role of genes thought to be factors in the pathogenesis of high blood pressure in the lungs. On the other hand, mice provide better genetic disease models. They are useful for screening for drug effects quickly and efficiently before embarking on experiments with longer durations and more severe diseases using rat model(s). Rats, however, develop lung disease in a way that is even more similar to human pathology. Rats may also be used as it is necessary to have a second rodent model proposed here for pharmacological studies, i.e., to test the most promising therapies after they have been shown to work in mice.

In terms of techniques used, radiotelemetry allows longitudinal blood pressure measurements within homecage environment, and the same applies to a low-environment chamber in which animals stay in their own cage, with littermates, within the duration of the experiments.

The low-oxygen environment chamber is routinely supplied with soda limes and is regularly ventilated to prevent a build-up of CO2. Humidity build-up is rarely observed; when it is, an appropriate de- humidifier is used.

Why can’t you use animals that are less sentient?

We choose our models to provide adequate data whilst impacting animal welfare the least. However, we cannot use less sentient animals than rats and mice, as these are the lowest vertebrate groups in which models of high blood pressure in the lungs have been developed and are thus the most appropriate species to study. Mice are the easiest rodents to breed and maintain in larger quantities (as opposed to guinea pigs or mole rats, for example).

Generating genetically altered mice allows disease mechanisms to be studied on a cellular and/or genetic level. Young and adult mice provide better genetic models of disease as we can breed animals with specific defects in their genes, which are involved in particular pathways underlying disease and are thus helpful to screen for drug effects quickly and efficiently. However, we cannot employ less sentient neonatal mice for this purpose because these will not possess physiological and pathophysiological changes that usually occur during the progression of this disease in adults or younger people.

Rats may also be used as it is necessary to have a second rodent model to test the most promising therapies after they have been shown to be working in mice and before work can progress towards clinical trials in human patients. In both species, animals will be

terminally anaesthetised, and non- invasive and invasive measurements will be carried out under terminal general anaesthesia before culling animals and harvesting tissues for further assessment.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

One of the most important experimental models with this programme will involve the exposure of animals to a low-oxygen environment. As with humans, prolonged exposure to a low-oxygen environment may cause an increase in respiration, alterations in blood pressure and decreased locomotor activity. The refinement will include animals being closely monitored during exposure and returning them to room air permanently when showing limiting clinical signs, leading to immediate recovery.

Animals will be inspected and monitored at least daily for any signs of distress. If an animal shows any predetermined signs of illness or discomfort, it will be humanely killed.

All animals are humanely killed at the end of the experiments, and tissues are harvested for further analysis. We work closely with the veterinary surgeon, who advises us on methods to maximise animal welfare and minimise harm to animals in our experiments.

Surgical positioning of the blood pressure probe into the body's main blood vessel (i.e., aorta) or the heart has several advantages and considerable refinement over other more invasive surgical procedures. There is no necessity for restraint, sedation or heating of the animals as required by the technique of indwelling catheterisation, making the method more physiological and less stressful.

Monitoring is entirely remote, removing the stress of human contact whilst benefiting from continuous pulmonary artery pressure readings in the home cage.

Several of our protocols involve the assessment of lung vessel blood flow and/or pressure in the heart under terminal general anaesthesia. We intend to employ several other methods to assess responses to disease development or treatment based on clinically meaningful endpoints described in patients. These include non-surgical, non-invasive measures by echocardiography (also called ultrasound), cardiac magnetic resonance or computer tomography imaging. In collaboration with other Departments, we plan to develop an MRI technique that allows further refinement of non-invasive assessment of cardiopulmonary hemodynamics. These non-invasive physiological assessments will be followed by end-terminal procedures and post-mortem analysis of harvested tissues and plasma to maximize the data yield from each experimental subject without causing additional distress to the animals. These refinements of our animal phenotyping protocols will involve fewer animals to determine preclinical efficacy.

Many of the animals that we plan to use will be anaesthetised and humanely killed under anaesthetic without waking up (which does not cause any additional harm to the animals). Tissues will be collected at the end for an additional (genetic, biochemical or histological) post-mortem analysis. This will maximise the data collected from each animal used and inform further studies whilst animal numbers are minimised. When it is not possible to carry out these experiments without using animals, we will aim to reduce the number of live animals and instead perform experiments with isolated organs or cells.

Specific actions will be taken to reduce pain and suffering in the animals. Any surgical techniques will be performed by experienced licensees who are trained, follow good laboratory practice best practice of peri-operative care, and consult regularly with veterinary surgeons and skilled technicians to minimise any discomfort/distress. We prefer to employ inhaled anaesthetic in our experiments, which makes it easier to control the depth of anaesthesia without a profound impact on blood pressure and heart rate, and the use of appropriate analgesics to control pain will be employed.

Whenever possible, animals will be used at the earlier possible time point for an assessment, with the aim of minimizing the time they are studied.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Our group y plans all experiments well in advance, and we will continue to do so by referring to and conforming to ARRIVE guidelines for reporting in vivo animal research.

Experimental plans will be discussed (or advised) whenever possible between the licence holder, deputy, group members and other members of the Departmental community. We will also consult with the medical statistics specialists available within the Department to advise on the experimental design if data from the pilot experiments are provided. In addition, we regularly communicate with other scientists in the field, including our collaborators, during local, national or international meetings on better methodology or refinements and to ensure that the experiments are scientifically valid and performed in the best- refined way. Any surgery proposed here will be performed under general anaesthesia and using aseptic techniques that meet the Home Office Minimum Standards for Aseptic Surgery and LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2nd Edition, 2017 - https://www.lasa.co.uk/wp-content/uploads/2018/05/Aseptic-Surgery.pdf). All experiments will also be reported according to ARRIVE guidelines for reporting in vivo animal research.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have already been subscribed to the National Centre for 3Rs in Animal Research mailing list, and we regularly receive updates about conferences, meetings, new tools, and funding schemes. We will continue to visit the dedicated website (https://nc3rs.org.uk/) and speak to our local vet about new improvements and expectations.

We are an active member of the local animal facility community, which oversees the management and day-to-day running of the imaging equipment, the use of animals in research, related training and other local issues.

20. Evaluation of new lipid nanoparticles (LNPs) for delivery of vaccines and therapeutics

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Vaccines, mRNA, Therapy, Bacterial infections, Lipid nanoparticles

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The main aims of this project are 1) to characterise the ability of lipid nanoparticles to deliver therapeutics to specific organs, and 2) to develop vaccines for protection against pathogen diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Tiny fat packages, called lipid nanoparticles, have played an important role in the success of mRNA vaccines against COVID-19, unlocking their great potential in the delivery of therapeutics.

Being able to encapsulate mRNA (and other nucleic acids and therapeutic agents) for

delivery to specific organs would allow several types of diseases to be targeted, while minimising the side effects of the treatment. Tissue-targeted delivery of these particles is currently still a challenge. Our aim is to assess different nanoparticles for their ability to reach specific organs.

We are also working on the generation of vaccines against pathogens, with a growing interest for bacteria resistant to antibiotics. The World Health Organisation (WHO) has reported a worrying global rise in antibiotic resistance, which poses a significant threat for the treatment of bacterial infections, especially in less-developed countries. However, there are currently no mRNA vaccines against bacterial pathogens in clinical development.

What outputs do you think you will see at the end of this project?

The main outputs expected from this project are:

- data on the biodistribution of our lipid nanoparticles (LNPs): this means characterising their ability to encapsulate and deliver therapeutics to specific organs and how they are cleared by the body
- efficacy data from selected LNPs and characterisation of their ability to generate an immune response to inform the development of new vaccines that can be taken into future clinical trials

Who or what will benefit from these outputs, and how?

In the short term, the key objective is to evaluate LNP candidates as good targets to develop vaccines.

In the long term, identified LNP candidates will be further evaluated and eventually progressed into human clinical trials for delivery of therapeutics (vaccines and other applications, including cancer and inflammatory diseases - such as inflammatory bowel disease or Crohn's disease).

Our research will benefit both the scientific community and, ultimately, the general public.

How will you look to maximise the outputs of this work?

We have several academic partners with expertise in the field of lipid nanoparticles and/or in different pathogen-related diseases: they regularly take part in our scientific discussions and - wherever possible - our results are shared at academic seminars and eventually published.

We also actively participate in national and international conferences, where we discuss our data by giving a talk or presenting a poster.

We have a very openminded approach when it comes to sharing results, ideas and we are very keen to collaborate with the scientific community.

Species and numbers of animals expected to be used.

- Mice: 4000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult mice are the animals with the lowest neurophysiological sensitivity and a fully developed immune system, which allow us to extrapolate data useful to build a preclinical package to then justify clinical trials in human patients.

Typically, what will be done to an animal used in your project?

Animals will undergo injection of substances (up to three times), blood sampling (up to three times), non-invasive imaging, killing. Typically, experiments last between one and five weeks.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals will mainly experience some mild and transient discomfort when substances are injected and/or blood samples are taken, however there is the possibility <10% animals may experience moderate severity due to repeated brief inhalation anaesthesia or for certain routes of administration.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

>90% mild
<10% moderate

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Full evaluation of the biodistribution of lipid nanoparticles (LNPs) in the organism, as well as the efficacy of mRNA-LNP-based vaccines after administration, are parameters that need to be addressed in a living system. The complexity of the mammalian system cannot be recreated *in vitro* to ensure our results are relevant for future clinical trials.

Which non-animal alternatives did you consider for use in this project?

The transition of experiments into animal models follows an initial *in vitro/ex vivo* work to characterise that LNPs are: i) able to deliver mRNA to cells and that mRNA is efficiently translated into protein, ii) not toxic for human immune cells from healthy donors, iii) not triggering an innate immune overreaction. Only functional and 'safe' LNPs are tested in mice. No animal work is carried out where the desired information could be obtained via alternative methods: *in vivo* studies are designed around a deeper understanding of the biodistribution and the efficacy of vaccines of LNP being assessed. This limits the number of mice required under this licence but it cannot replace them entirely.

Why were they not suitable?

Some of the complex interactions we aim to address in a living system - which are not possible to address in non-animal alternatives - are listed below:

- The best route of administration (which can also be reflected later in clinical trials); this plays a role in determining the delivery of LNPs to different organs and how they are cleared by the body
- The immune response activation after vaccination

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the number of animals to be used based on similar studies carried out by academic collaborators and external companies. Both numbers of animals and doses have been optimised. We may use pilot studies with a small number of mice to assess the minimum number of animals required to achieve statistical relevance.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We rely on published guidelines and we follow the vaccination schedules with the indicated numbers of animals per group. For new experiments, group sizes are determined after consultation with biostatisticians.

To minimise and control the source of variability, we use similar group sizes across different experiments and we use standard operating procedures: for instance, we use validated methods for substance preparation, and standardised imaging procedures.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Pilot studies will be performed to optimise new elements before running a bigger study -

for instance when a new combination of nucleic acids (encoding different antigens) is encapsulated in the LNPs. Pilot studies will be designed with fewer animals (reduction) and more controls groups, but they will still be designed in a way that will allow us to use the generated data (combined with data from follow-up studies).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

During this project we will be mainly using adult wild-type mice. In some cases, genetically modified mice will be required: these are reporter mice (which allow to track changes in specific cell types and organs) that will not cause any harms.

Animals will undergo injections of substances, blood sampling and non-invasive imaging.

The administration will be mostly through standard routes that cause only mild and transient pain and discomfort. Different routes are required as these can influence organ distribution, and therapeutic outcomes.

Why can't you use animals that are less sentient?

A full evaluation of the biodistribution of lipid nanoparticles in the organism and analysis of the specific immune response to vaccines are parameters to be addressed in a mammalian system. Mice are the species with the lowest neurophysiological system that allow us to extrapolate data useful to build a preclinical package to then justify clinical trials in human patients.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

- Treatment will always be delivered at the smallest effective delivery volume which is not expected to cause any clinical symptoms but allows us to achieve our scientific aim
- Recovery from anaesthesia will happen in a heated recovery box under close monitoring
- Best practice will be followed for every protocol

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow guidance from the Laboratory Animal Science Association. We will also work to the ARRIVE and PREPARE guidelines

How will you stay informed about advances in the 3Rs, and implement these

advances effectively, during the project?

We aim to attend animal welfare meetings held with subject experts where best practice on the 3Rs is shared.

We will sign up to the NC3Rs newsletter.

Also we will monitor the following webpages: Norecopa, Research Animal Training (RAT), RSPCA.

Home Office

21. Uncovering the unusual mechanisms of malaria parasite sexual development

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Malaria, Plasmodium, Sexual development, Cell biology, Transmission

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

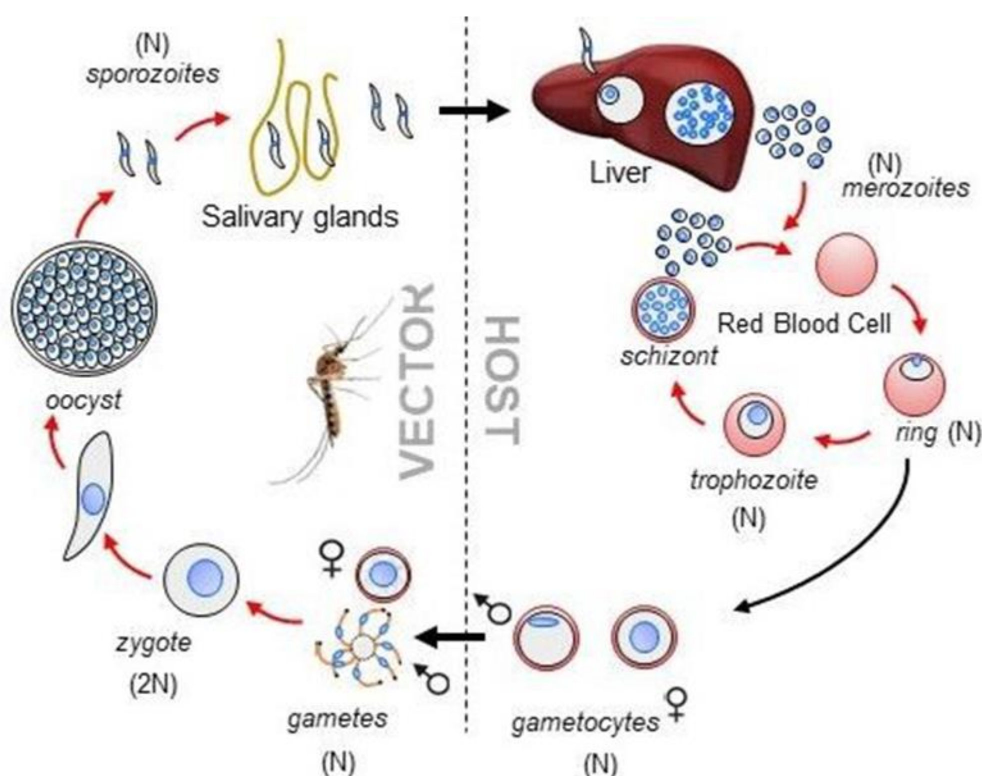
What's the aim of this project?

To uncover fundamental knowledge that drives the highly divergent process of sexual development in the malaria parasite
Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Despite much progress been made towards controlling malaria over the last fifteen years, the parasite (genus Plasmodium) still infects a quarter of a billion people and claims over 400,000 lives every year. Compounding this, resistance to current therapies to treat malaria infection (including chloroquine or hydroxychloroquine) is advancing at a rapid pace. This project license will enable hugely important research into the highly divergent processes that govern malaria parasite development across its entire life-cycle, which occurs within both mammalian host (asexual stage) and mosquito vector (sexual stage). This project’s main focus is on the sexual stages, but may also encompasses all other life-cycle stages.

Current research into the most lethal human-infective malaria parasite (Plasmodium falciparum) is limited since it is difficult to study sexual stages in the mosquito. Hence, this project will utilize the well- established mouse model of malaria (Plasmodium berghei), which will enable us to analyze the whole life-cycle in the laboratory and is highly reflective of the human-infective P. falciparum. This is hugely important since the sexual stages are responsible for its transmission, and several phases of cell division occur in this stage. Uncovering the novel mechanisms that drive malaria parasite sexual development will also be of huge benefit to researchers in medicine, industry and academia since this could accelerate drug development by uncovering novel targets for therapeutic intervention, while providing a platform for future research towards further understanding of the basic biology behind malaria parasite sexual development.



The malaria parasite life cycle occurs in both a mammal host and in the mosquito. Replication without sex occurs in the liver and bloodstream of the mammal (right side of the figure) and sexual reproduction occurs in the mosquito (left side of the figure) in many stages.

What outputs do you think you will see at the end of this project?

Outputs will be data, which can be presented in the format of publications/seminars/posters at conferences and press releases on the mechanisms of various malaria sexual development processes, or on new therapeutics to treat the disease.

Who or what will benefit from these outputs, and how?

This project will benefit researchers in medicine, industry and academia because:

(1) Establishing the divergent mechanisms of Plasmodium sexual development and the roles of the key genes involved in the rodent malaria model, P. berghei will have direct relevance to human- infective P. falciparum. Thus, the data generated will be of significant value since the P. falciparum sexual stages are difficult to study.
(2) Determining the key functions of genes important for sexual development will uncover potentially novel candidates for inhibiting malaria parasite transmission. Hence, our research will provide important targets to those developing new therapeutics or vaccines against the parasite.
(3) The transgenic parasites generated during this project will provide a unique resource that will be available to researchers both in the UK and globally.
(4) This project will not only significantly influence the field of parasitology. The project will potentially stimulate further academic research into cell division of other parasite species that have huge socioeconomic impact on livestock and humans globally, including Toxoplasma spp., Eimeria spp. and Cryptosporidium spp.
(5) The knowledge derived from this project will benefit academics from other disciplines e.g. evolutionary biologists, cell biologists, biochemists, protein chemists and pharmo-bioscientists who work on parasitology, vector-bourne diseases, evolutionary divergence, chromosome dynamics, and protein structure/function. The data of benefit to other researchers will be made available through publication in peer-reviewed high impact publications, presentation at conferences and deposition of public repositories such as PlasmoDB and RMgmDB. There is a vibrant community of researchers in vector-borne diseases in the UK and elsewhere, thus there is an audience highly receptive to the new insights this project will bring.

Project data will be published, with suitable annotation and experimental details, in open access journals and/or on the PI's lab website to allow it to be easily accessed and used by researchers in the field. All other data will be made available through to various databases established for the malaria research community, such as PlasmoDB and the RMgm database.

How will you look to maximise the outputs of this work?

Outputs will be published, including unsuccessful results in data repositories where possible. Outputs will also be presented at conferences where appropriate, collaborations formed and information shared where appropriate.

Species and numbers of animals expected to be used

- Mice: 600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Six-to-eight-week-old (i.e. adult) female, wild-type mice will be used for the project as is standard practice in the malaria field. Females are used because physiological levels of oestrogen, rather than progesterone, enhance immunity and protect females from severe disease symptoms such as cerebral malaria during infection (Klein et al. 2008 Gender

Medicine Volume 5, Issue 4, Pages 423-433), which is reflected in humans (Bernin, 2014. The Journal of Infectious Diseases, Volume 209, Issue suppl_3, July 2014, Pages S107–S113) and we want to avoid severe cerebral malaria infection. In addition, the use of six-to-eight-week-old mice is required since malaria infection in pups results in stunted growth and a higher chance of death, along with decreased behavioural scores compared to controls and infected adults (Smith et al. 2022. Parasite Immunology, 44(12):e12952), which again we want to avoid. Mice are essential for propagating the rodent malaria model P. berghei and offer the perfect environment in which to clarify in vivo the mechanisms of blood stage development and allow transmission to mosquitoes for analysis of cell division during vector stages.

Typically, what will be done to an animal used in your project?

For growth of parasites in mice and drug selection:
For initial infection, mice will be injected intraperitoneally with drugs to increase production of red blood cells up to two days prior to infection, which increases the chance of malaria infection and therefore reduces the need to increase the number of mice required to produce high-quality data. Approximately twenty-four hours post-infection, mice infected with blood containing either wild-type or genetically- modified malaria parasites (used to study important genes required for parasite development) will undergo antiparasitic drug selection (provided in the drinking water and will select for parasites containing modified genes) for up to 9 days. Mice infected with wild-type parasites (necessary for use as a control) will not undergo drug selection but may undergo drug treatment if experiments require selection of pure gametocyte (sex cell) populations. During this time, mice will not normally show any signs of infection. On approximately day 10, blood smears will be taken from one drop of superficial vein blood (up to 20 ul) to monitor parasite numbers (parasitaemia). If parasitaemia is between 2%-15% of the total red blood cell count, mice will undergo terminal anaesthesia and cardiac puncture to retrieve infected blood. If parasitaemia is below 2%, parasitaemia will be monitored using superficial vein blood daily until parasitaemia reaches 2-15%, at which point mice will be humanely killed and undergo cardiac puncture to retrieve infected blood.
For transmission experiments:

For transmission of malaria parasites from mice to mosquitoes, infected mice will undergo terminal anaesthesia and once anaesthesia has been confirmed, mice will be placed on top of a cage holding up to 50 mosquitoes, which will feed for a maximum of 15 mins. This will be critical for study of malaria parasite development in the mosquito.

For transmission of malaria parasites to mice, two days prior to infection, uninfected mice will be injected intraperitoneally with drugs to increase production of red blood cells up to two days prior to infection. On the day of infection, mice will undergo surgical anaesthesia (Plane 1) and once anaesthesia has been confirmed, mice will be placed on top of a cage holding up to 50 mosquitoes, which will feed for a maximum of 15 mins. Mice will then be returned to their holdings and continually monitored for at least one hour, or until full consciousness (assessed by observed normal behaviour) has been achieved. Four days post-infection, blood smears will be taken from one drop of superficial vein blood to monitor parasitaemia. If parasitaemia is between 2%-15%, mice will be humanely killed, and infected blood retrieved via cardiac puncture and used for other experiments.

What are the expected impacts and/or adverse effects for the animals during your project?

Adverse effects are usually observed in response to high parasitaemia (>15%; Huang et al. 2015 Bio Protoc. 2015 Jul 5; 5(13): e1514). Primarily, adverse effects observed will be increased respiration, diarrhoea, hunched posture, piloerection, lethargy and reduced locomotor activity. Weight loss may be seen in some mice.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The animals undergoing infection will reach a maximum of moderate severity level if parasitaemia rises above 15%. This is expected to occur in <10% of mice. The remaining mice are expected to show mild adverse effects due to infection, or mild transient discomfort from the procedure.

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Mice are crucial for studying P. berghei as a model since culturing many parts of the life-cycle in vitro is very difficult or not possible at all. This is especially true for the human infective P. falciparum. Hence, use of the rodent model offers an established way of analysing the entire malaria life-cycle in vivo that is highly reflective of the human infective parasite. However, for mosquito breeding, we will explore replacement of mice with artificial blood meals (Marques et al., 2018. Scientific Reports. 8, 17807).

Which non-animal alternatives did you consider for use in this project?

Cell culture and human malaria models. For mosquito breeding, we will also explore the possibility of using artificial blood meals.

Why were they not suitable?

Cell culture of the vertebrate stages of the malaria life-cycle in both rodent (P. berghei) and human models (P. falciparum) is extremely difficult to perform and does not represent the entire life-cycle. Using mice and the rodent malaria parasite is a robust in vivo model allowing study of all developmental and proliferative stages of the life cycle in the mammalian host and mosquito vector. Most importantly, it provides access to sexual stages in the mosquito that are difficult to study for human malaria parasites such as P. falciparum. Studies in P. falciparum require high containment facilities for the mosquitoes, and a model for bite-back studies, liver development and subsequent blood stages requires use of primates. P. berghei is not zoonotic and does not require high containment.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals we will use has been calculated based on the minimum numbers required to achieve each aim. Overall, the project aims to investigate the key roles of genes involved in malaria parasite sexual development.

The first aim is to characterise the important functions of up to 6 proteins involved in parasite sexual development throughout the life-cycle. This will involve deleting each gene, at least three times and growing the parasites in mice to determine their key functions. Generation of clonal genetically modified parasites and the analysis of their function will require approximately 200 mice.

The second aim will look to study when and where certain proteins important for parasite sexual development are needed. This will utilise microscopy for this to study proteins tagged with a fluorescent protein throughout the parasite's life-cycle. We aim to analyse up to eight proteins, each being repeated three times. It is estimated that 200 mice will be required for this aim.

The third aim will be to determine how key proteins involved in malaria parasite sexual development interact with each other. This will be achieved by using proteins tagged with a fluorescence protein, and interactions studied using a procedure called proteomics. We aim to study several tagged proteins, which require up to 10 mice per experiment in order to obtain enough material for the experiment, along with wild-type parasite controls. It is necessary to repeat each experiment three times in order to obtain statistically significant data. Overall, it is estimated that this aim will require approximately 200 mice, but will endeavour to reduce the numbers required should better techniques become available.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The lead PI has undergone training in experimental design and also received statistical advice prior to applying for funding for this project. To ensure confidence and statistical significance, all experiments will be performed two to three times and at all times the 3Rs principles and ARRIVE guidelines will be strictly applied.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will look to reduce the number of animals required to generate material for all experiments in consultation with expert collaborators. Technology is continually improving and becoming more sensitive year on year, and we will continually assess this throughout

the project. We will also keep abreast of publications that refine methodologies according to 3Rs principles, and adopt these as soon as possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are crucial for studying P. berghei as a model since culturing many parts of the life-cycle in vitro is very difficult or not possible at all. This is especially true for the human infective P. falciparum. Hence, use of the rodent model offers an established way of analysing the entire malaria life-cycle in vivo that is highly reflective of the human infective parasite. The maximum severity level set for this project is moderate, since mice will be humanely killed prior to parasitaemia reaching up to 15% to reduce Pain, Suffering, Distress or Lasting Harm as much as possible. A maximum severity level of moderate will be more than sufficient to achieve the scientific endpoints of the study.

Why can’t you use animals that are less sentient?

The rodent malaria parasite requires live mice in which to propagate during the asexual phase of the life cycle, which lasts over several days and therefore terminally anaesthetised mice cannot be used. Sexual stages occur within the mosquito, and will be used for this stage. Other species that are regularly used for malaria models are primates, which we do not wish to use. In addition, although P. berghei can infect rats, our previous studies have utilised mice to produce robust data.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Procedures will be regularly reviewed to ensure minimal welfare costs for the mice. Mice are housed already in appropriate cages that encourage species-specific behaviour, and are regularly monitored to ensure their welfare. We will also ensure correct analgesia and anaesthetics are appropriately used, and post-procedure for transmission experiments mice will be continually monitored until they are fully conscious.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All experiments will be performed according to strict compliance to PREPARE and ARRIVE guidelines (Du Sert et al, 2020 and Norecopa website) as set out in “Smith (2020) Guidelines for planning and conducting high-quality research and testing on animals. Laboratory Animal Research volume 36, Article number: 21”. We also have established procedures in place (Guttery et al, 2014; Cell Host Microbe; 16(1): 128–140; Zeeshan et

al, 2023; Nature Communications; 14(1):5652) that adhere to these guidelines rigorously. Additionally, for administration of substances, the following references will be regularly consulted and processes reviewed accordingly: Administration of Substances to Laboratory Animals: Routes of Administration and Factors to Consider - PMC (nih.gov), Administration of Substances (researchanimaltraining.com), Refining procedures for the administration of substances (sagepub.com).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Regular discussions will be held with the expert Named Persons at the establishment and animal technicians to review current approaches and whether there are any new 3Rs opportunities. We will also keep abreast of relevant publications, subscribe to the NC3Rs newsletter and attend relevant seminars and workshops. Methods that may significantly alter the way we perform our experiments will be adopted as soon as possible in consultation with, and guidance from all Named Persons at the establishment.

22. Breeding Maintenance and Production of Genetically Altered Mice

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Maintenance of genetically altered or mutant mice, Medical Research, Breeding

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to support basic and translational research by providing genetically altered or mutant mice for research projects by breeding and maintenance of established genetically altered mice.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This work will underpin a range of research projects directly enabling many significant and high-impact research findings, that increase our understanding of several research fields including, but not exclusively, to human disorders ranging from infertility and cardiovascular disease to rare, debilitating, genetic conditions.

What outputs do you think you will see at the end of this project?

This project will underpin a range of scientific studies, from basic research to translational projects for example, cardiovascular, infertility and other genetic conditions. This will look like the production of mice which will be transferred to specific projects.
The scientific insight underpinned by this project will lead to greater understanding in the relevant field leading to research publications, greater information for individual patients or patient groups, and provide resources that will benefit the wider scientific community.

Who or what will benefit from these outputs, and how?

Beneficiaries of these outputs will be - the wider research community, individual patients and patient groups, local researchers and their international collaborators.

Direct benefits include:

- the maintenance of high quality GA (Genetically Altered) animals using standard protocols to enable researchers at (or moving to) the establishment to continue their research effectively and efficiently.
- Facilitating international collaborations and interactions to significantly enhance research output.
- This licence enables specific colony manager oversight of breeding, implementation of consistent processes and best practice.
- The focus and support of this licence may change depending on the type of studies being supported.

How will you look to maximise the outputs of this work?

The single licence supports a centralised process. This enables collaboration and resource sharing (e.g. tissue from excess stock), furthermore there is a rigorous selection process during which each individual project is scrutinised to ensure that the most appropriate and refined methods are employed and that the GA animal has the greatest likelihood of achieving the desired goals.

Species and numbers of animals expected to be used

- Mice: 15000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are frequently used to model human disease. As such, they are the most appropriate species due to well-established protocols that allow accurate genetic manipulation from 1

base pair, a fundamental unit of DNA (Deoxyribonucleic acid, the molecule that carries the genetic information for the development and functioning of an organism), to much larger, more complex genetic changes.

The life stages required for breeding and maintenance on the project will include all stages of development; embryo, neonate (e.g., newborns), juvenile to adult and pregnant females. This is required to ensure we have the correct life stages available to enable production and to allow analysis of the effects of modification at a tissue level.

Typically, what will be done to an animal used in your project?

The majority of the mice will be bred to maintain the genetic alteration using standard breeding practices, either as breeding pairs or trios of 1 male with 2 females. This breeding will in most cases be continuous, and females will have up to 6 litters in their breeding lifetime.

Some wild-type animals (i.e., not carrying the genetic alteration of interest) will be used in breeding with genetically altered (GA) or mutant mice to refresh the genetic background of those lines as per recognised best practices.

What are the expected impacts and/or adverse effects for the animals during your project?

The vast majority of mice under this licence are not expected to show adverse effects and are only used for breeding to maintain genetic alterations.

A proportion of genetically altered mice will show some features of the human diseases they are designed to model, for example, abnormalities of the liver, kidneys, heart, eyes and brain and other body systems.

Animals on the mild breeding and maintenance protocol would be expected to experience no more side effects than a standard wildtype animal in natural mating, in some cases they will require a small tissue sample or blood sample to be taken for confirmation of gene presence/absence (genotyping).

Animals on the moderate breeding and maintenance protocol may exhibit abnormalities synonymous with the conditions they expect to model, details of which will be on a strain by strain basis. Where appropriate, the initiation of these adverse effects may be controlled by an inducible genetic promotor (region of DNA that switches the gene/DNA of interest on or off). In some cases they will require a small tissue sample or blood sample to be taken for confirmation of gene presence/absence (genotyping). On a case by case basis (rarely) this small tissue sample may need to be repeated.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild Breeding and Maintenance: 60% of animals in the project will undergo this protocol (embryo, neonate, juvenile, adult, pregnant)

Moderate Breeding and Maintenance: 40% of animals in the project will undergo this protocol (embryo, neonate, juvenile, adult, pregnant).
The majority of these animals will experience no more than standard/normal animals in breeding and their experience will be sub-threshold.

What will happen to animals used in this project?

- Killed
- Used in other projects
- Kept alive at a licensed establishment for non-regulated purposes or possible reuse

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The investigation of single and multiple gene effects can be undertaken in a variety of in-vitro (e.g. not using a whole live animal) and non-mammalian systems and a variety of these will be utilised where appropriate. However some studies require the use of live animals, in order to investigate gene effects in a whole complex mammalian organism.

Which non-animal alternatives did you consider for use in this project?

Cells cultured in the laboratory and computer-generated models are widely used by the research teams supported by this application to complement animal studies, however, these are not an absolute alternative as it is not possible to model the effects of a disease gene on the whole body by only using cells in culture.

Why were they not suitable?

Standalone cells cultured in the laboratory are not suitable because they act differently in-vitro and the use of embryoid bodies (i.e., 3D aggregate of stem cells) and 3D modelling are of limited capacity, especially when assessing whole body effects or in the context of complex organs.

Cells, embryoid bodies, etc do have a role but are currently not suitable as replacements. Computer generated models do not have the accuracy to predict whole body effects of small or large genetic changes due to the complex interactions of body systems.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated numbers are based on previous demand for the maintenance of genetically altered mice.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Holding a single project licence to breed these small colonies enables control of the numbers bred by utilising suitably designed breeding strategies. We ensure uniformly high standards of animal care and welfare are applied as all staff involved have extensive experience in this field. We employ strict colony management to maintain the genetic integrity of our colonies and as such reduce wastage. We enable effective sharing of some colonies between different research groups, to allow a reduction in the number of animals produced. This is facilitated by the in-house colony management team, who manage all aspects of mouse breeding.

The Assessment Framework for Efficient Breeding of Genetically Altered Animals will be utilised to promote efficiency throughout the term of the licence.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Breeding colonies will be monitored carefully to avoid over-production of animals, and animals of specific genotypes that are produced will be used by more than one research group whenever possible.

Breeding colonies that are not required in the short/medium term will be stored as frozen gametes, to minimise continued production of GA animals. Where desired animals are readily available from external sources (with compatible health status), mice will be acquired. This avoids duplicating generation and may also avoid unnecessarily maintaining breeding colonies.

Furthermore, we actively participate in a number of projects for archiving and sharing of mouse lines and phenotyping information (eg EMMA, MRC Mouse Network) as well as maintaining informal arrangements for sharing lines such as tissue-specific cre lines.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Genetically altered mice are proven to be a powerful way to model and begin to understand human disease. This provides insight not possible from other sources.

Methods used are:

- **Natural matings of GA, mutant and wildtype mice in pairs/trios (**strain dependant efficacy): no lasting harm or change in experience from an animal maintained standardly. In some cases they will require a small tissue sample or blood sample to be taken for confirmation of gene presence/absence (genotyping). Most common method used will be ear notch (carried out for identification purposes - the tissue being utilised for genotyping) which causes minimal, transient pain. Re-sampling will only occur when confirmation of genotype has not been achieved or identification is compromised. Secondary methods such as tail tip or blood sampling would only occur with scientific justification where ear notch is insufficient.

Why can't you use animals that are less sentient?

Whilst less sentient animals such as zebrafish can provide some insights into basic aspects of vertebrate research and are used to complement mouse studies, a mammal is more appropriate to better understand conditions affecting complex tissues and organs/systems.

Wherever possible, the earliest stage of development will be used; however, embryonic stages are not suitable for the study of conditions such as liver fibrosis (e.g., thickening or scarring of the liver), kidney disease, heart/circulatory function, etc.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The majority of genetically altered and mutant animals bred under the authority of this project have no clinically harmful characteristic or can be maintained with only one gene copy (i.e., heterozygous) or turned on/off by another gene (by administration of a substance, which would not be carried out on this licence) so that clinically significant adverse effects are not apparent. Homozygote (i.e., having two identical copies of a particular gene or genes and exhibiting the resulting characteristic) animals with an evident characteristic are produced only when specifically required for the project or, if produced during a breeding programme, are humanely killed as soon as possible if the characteristic affects welfare. Some adjustments to husbandry will be made to support animals that may have minor welfare impacts (e.g. longer water bottle nozzles for muscular dystrophy mice, teeth trimming for models of dwarfism etc.)

Where these evident characteristics only develop after several weeks, breeding programmes will be used to utilise younger animals, which are euthanised before the characteristics develop.

When a new GA line is to be imported for maintenance on this licence, details of the anticipated characteristics will be obtained from the supplier, and this will inform the husbandry conditions/modifications as outlined in Wells et al., 2006, e.g. barrier/isolated conditions for animals with a depleted immune system, diet/bedding requirements etc. for optimal line maintenance. This information may be supplied as a "mouse passport", if not, this will be complied with and documented as soon as possible.

Genotyping will normally be undertaken using tissue removed when ear-notching for

identification purposes. When this is not suitable, blood sampling may be used, and in some instances a tail-biopsy may be required. Re-sampling will only occur when confirmation of genotype has not been achieved or identification is compromised. Secondary methods such as tail tip or blood sampling would only occur with scientific justification where ear notch is insufficient.

Any pain associated with ear notching for genotyping is transient and minimal. For other methods pain will be minimised by appropriate use of analgesia.
Furthermore, as standard non-aversive handling methods will be used to reduce stress, additional in- cage enrichment, the inclusion of "houses" within the home cage provides a more secure environment for breeding animals to make their nests, and improved bedding that we find reduces neonatal losses.

Cryopreserving (i.e. cooling and storing cells/tissues at a very low temperature for future use) animals where appropriate e.g. earliest opportunity after import of new strains or after backcrossing (i.e., mating on related and unrelated individuals) will be used to ensure strains are preserved with a minimal amount of random genetic changes (also known as genetic drift) and therefore preserving their integrity.

Backcrossing of mouse strains at appropriate points such as every 5-10 generations, dependent on colony size and backcrossing of both sexes to ensure good refresh of both sex chromosomes (e.g., X & Y chromosomes), will preserve genetic health and the increased reproducibility by limiting genetic drift that occurs naturally across generations. Utilising Single Nucleotide Polymorphism (SNP: a variation in genetic material occurring when a single nucleotide (building blocks of genetic material) at a particular site in the genetic material differs between members of a strain) analysis to ensure correct back crossing to preserve genetic health. Analysis will occur either at cryopreservation and/or before backcrossing occurs to establish the correct choice for background. In exceptional circumstances backcrossing will not be appropriate and working with the colony management team we will find alternative approaches to support preservation of genetic integrity.

Utilising the breeding system that is most suited to the efficient production of that strain of mouse, this will be based on data or information available in mouse passports e.g. pairs/trios.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Alongside the guidelines listed below, I will also adhere to local AWERB standards for research animals, and where appropriate, support the development of new local standards for refinements discovered during the project licence.
Code of Practice for Housing and Care of Animals Bred, Supplied or used for scientific purposes

- LASA Guidelines
- RSPCA Animals in Science guidelines
- UFAW Guidelines and Publications
- NC3R's and Procedures with Care

I will consult with the Colony Manager to review genetic health, breeding practices and

overall colony health and management at regular intervals.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The local AWERB (Animal Welfare and Ethical Review Body), NIO (Named Information Officer), NACWO (Named Animal Care and Welfare Officer), NTCO (Named Training and Competency Officer), Colony Management and Veterinary team regularly inform, and disseminate improvements and recent studies involving reduction, replacement and refinement

Alongside external resources including (but not limited to); collaborators, peers, conferences and lab animal and animal welfare bodies.

During the 1, 3 and 5 year review of the project licence I will update on implementation or consideration of the 3Rs that has occurred during the previous period, alongside a review of the linked training plan, score sheets etc. in collaboration with the NACWO, NTCO, NIO, Colony Management and Veterinary team with a particular focus on refinements.

As a member of our local AWERB, and 3R's advocate I participate in numerous discussions and briefings concerning advances in the 3Rs. For specific techniques and breeding schedules I have a colony management team who attend regular continued professional development and are members of relevant professional bodies.

23. Investigation of Cryptosporidium Host-Pathogen Interactions

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

infectious parasitic disease, immunity, disease prevention, parasite biology, vaccination

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand how the host immune system recognizes and responds to a Cryptosporidium infection, how the parasite has evolved to evade this host immunity, and how environmental factors influence infection.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cryptosporidium is a major cause of diarrheal disease and causes significant mortality, morbidity, and developmental stunting in children around the world. Annually, there are an estimated 200,000 deaths and over 10 million disability adjusted life years attributable to Cryptosporidium infection. Despite this significant impact of public health, there are no fully effective drugs or vaccines available and the basic knowledge to drive their development is scant. Simply put, we don’t understand how the immune system responds to

Cryptosporidium infection. Understanding these basic mechanisms will help us develop new therapeutics. To understand the immune system a model is required where 1) there is an immune response comparable to what is seen in humans, and 2) this immune response can be modulated to test hypotheses. With the mouse model of cryptosporidiosis we have a natural infection that mimics human disease where we can dissect the immune response to understand the mechanisms that lead to long lasting immunity and protection.

What outputs do you think you will see at the end of this project?

The goal of this project is to develop a better understanding of the host-pathogen interactions of the Cryptosporidium parasite. From the perspective of the host, we aim to identify the genetic and environmental factors that play a role in resistance and long-term recovery from infection. From the perspective of the parasite, we aim to identify the virulence factors that the parasite uses to evade host immunity and cause infection. Through these studies we will also determine whether vaccination with certain parasite antigens can protect the host from infection and subsequent disease. Overall, we hope to broaden the foundation of knowledge that we can use to develop better therapies to treat and prevent infection. We will communicate our research as frequently as possible through public engagement and presentation, scientific conferences, and publications.

Who or what will benefit from these outputs, and how?

The Cryptosporidium research community will greatly benefit from the research we will perform. The scientific community at large will also benefit, to a lesser extent, as we hope that our research will uncover immunity and virulence mechanisms relevant to other fields. We will regularly publish our findings and communicate our research to the public. Cryptosporidiosis is a serious public health concern and we will do our best to advance the science, train new researchers, and educate our community about the disease.

How will you look to maximise the outputs of this work?

During the project we will encourage visiting scientists and students to come and learn techniques that we use and develop. We will also make these methods and protocols available through publications whenever possible. Data will be published in a timely manner and presented often to the wider scientific community during various conferences.

Species and numbers of animals expected to be used

- Mice: 7000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We use a mouse model of cryptosporidiosis for three important reasons:

1) To mimic human disease and vaccination, 2) investigate infection and immune response in a natural murine model, and 3) to propagate Cryptosporidium parasites.

The ability to mimic human disease in a rodent model is an invaluable tool that will help us to understand disease pathology, immune response, and develop better therapeutics. Human disease typically occurs in the first 10 years of life and thus neonates, juvenile and adult mice are infected for this research (1 week to 2 months of age). Experimental data and epidemiology indicate that diet is an important environmental factor that influences infection. For instance, the concentration of the bacterial metabolite indole in the intestine is linked to resistance to infection. Some mice will be fed controlled diets before and during infection to better understand how to control and treat disease.

There is no cell culture system to propagate Cryptosporidium, therefore, to maintain the parasite lines that we use for both in vivo and in vitro research we must use mice. Some species of Cryptosporidium, especially those that naturally infect humans, do not reproduce well in healthy mice, thus adult immunocompromised mice are required for propagation.

Typically, what will be done to an animal used in your project?

Mice are required for comparative infections and propagation of Cryptosporidium parasite strains.

For comparative infections, mice may be immunized against Cryptosporidium, given an alternative diet, or treated with antibiotics prior to infection. Mice are then inoculated with Cryptosporidium parasites by oral gavage. Infection will then be monitored indirectly via collection of faecal material or directly via whole animal imaging. The typical comparative infection lasts around 1 month and then mice are humanely killed. Intestinal tissue is usually harvested and stored for future applications, such as histopathology.

For propagation of parasites, mice will also be inoculated with Cryptosporidium through an oral gavage. Faecal material will then be collected for up to four months during the peak parasite shedding period of infection. At the end of this collection period mice are humanely killed.

What are the expected impacts and/or adverse effects for the animals during your project?

Most mice do not develop overt symptoms and recover from infection in 2-3 weeks. Highly immunocompromised mice occasionally show more severe illness and symptoms such as bloating and gastrointestinal discomfort; if any of those symptoms appear mice will be humanely killed. We do not expect any adverse reactions from vaccination or dietary adjustment.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Based on our previous experience, the severity is mostly subthreshold (60%), or mild (38%), with very few moderate (2%).

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The use of animals for project is required for two main reasons:

1) Mice serve as a naturally infected host in which we can study both parasite virulence and host susceptibility. To develop new therapeutics for this organism we require a better understanding of parasite biology and host immunity. Mouse models of infection allow for both.

2) There are no reliable and reproducible methods to propagate Cryptosporidium strains in vitro. Therefore, mice are required to maintain wild-type and transgenic strains required for all research that we do.

The Cryptosporidium research field is relatively new and, unfortunately, quite small. We rigorously review all newly published data within our field to ensure we do not duplicate any studies unnecessarily

Which non-animal alternatives did you consider for use in this project?

Before any experiment is designed, we search the scientific literature for similar studies that can either guide our design or partially answer the question we are intending to ask. We also review publications and data repositories such as https://data.jrc.ec.europa.eu/collection/id-0088, https://norecopa.no/alternatives/, and https://www.3rsinfohub.de/index.html for suggestions of any available alternatives to animal work.

For most experiments, we use in vitro models of infection. This includes infection of immortalized intestinal epithelial cells and intestinal organoids. Intestinal organoids are stem cells that differentiate into a variety of different epithelial cell subtypes that mimic the diversity that is naturally found within the intestine. Our lab maintains several organoid lines from humans and cows. There are many mechanisms by which intestinal epithelial cells recognise and respond to infection and organoids allow us to model this response without the murine model. Intestinal organoids also allow us to study late stages of parasite development. While the full life cycle of the parasite can occur within organoids, infections die out after a few rounds of replication and therefore it is currently not possible to propagate the parasite using this system. Therefore, for propagation of Cryptosporidium parasites or when studying host immunity, we use the mouse model of infection.

Why were they not suitable?

The immune system is very complex and we are still not able to mimic that complexity with in vitro systems. For propagation of parasites, part of the focus of my lab is to investigate ways to allow for a continuous propagation from in vitro culture, but it is impossible with the current technology.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

For comparative infections, sample sizes are determined using power calculations from previously available data. If there is no previously available data, then we commonly employ a pilot screen to measure the effect size in order to effectively calculate sample sizes. To minimise variability, we work with littermates. If littermates are not available, then mice are cohoused prior to infection whenever possible to limit any experimental differences due to microbiome. We also age match mice in control and experimental groups to minimise variability. Cages and collection samples are blinded prior to analysis to avoid bias. We have never seen a difference in infection from males to females and will use a combination of the sexes for our experiments. This limits the number of mice required for breeding. At the end of comparative infections, we commonly harvest intestines for fixation and storage, this allows us to use banked samples for DNA/RNA/Protein analysis and histopathology.

For propagation of Cryptosporidium parasite lines, the number of mice has been estimated based on the number of lab members and the annual usage rate from previous years. Again, both male and female mice will be used to limit the amount of breeding required.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Whenever possible we use organoid-based infection systems to model infection. The organoid system allows for us to examine Cryptosporidium infection with an in vitro system that has diverse epithelial cell types and architecture that more closely resembles the intestine.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Our lab developed a mouse model that supports long-term infections in mice without any increase in adverse effects. This led to an overall reduction in the total number of mice required for propagation of parasites. We also work closely with our colony managers to adjust breeding based on historical data, for example decreasing breeding in the late fall to anticipate holiday leave.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

In this project mice are used to model Cryptosporidium infection and to propagate parasite strains, which are essential to our research. Each step has been optimized to reduce stress and suffering. Cryptosporidiosis is not a disease with a sudden onset, meaning that even highly immunodeficient mice have a slow progression of symptoms. All mice are very closely monitored throughout infection and if any symptoms of discomfort are detected we immediately kill the animal and take intestinal samples for analysis. This allows us to reach our scientific goals while minimising the discomfort for the mice.

Why can't you use animals that are less sentient?

The mouse model allows for exceptional control over host genetic factors, which is crucial to studying the immunity and pathogenesis of the disease. The mouse model also offers a wealth of information and knowledge that we can use to guide our research. With this model we can investigate how the immune system recognizes and responds to infection, which can lead to new avenues of treatment and prevention. Put simply, less sentient animals will not have an immune response that we can effectively study.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will work closely with the veterinary and animal care staff to ensure that we are always refining our protocols to minimize harms for the animals we work with. Where applicable we may minimise use of wire mesh cage floors (which is used to allow collection of faecal samples) replacing them with any suitable bedding.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will stay up to date with the best practice guidelines developed by the National Centre for the Replacement, Refinement, & Reduction of Animals in Research, including their experimental design assistant https://nc3rs.org.uk/our-portfolio/experimental-design-assistant-eda. and the scientific literature for estimation of sample sizes based on power calculations. We will also refer to PREPARE guidelines when designing experiments and follow best practice described in https://researchanimaltraining.com/article-categories/procedures-with-care/ , When reporting our findings, we will follow the recommendations of the ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will keep up to date on the latest Cryptosporidium scientific literature and the latest news/research from NC3Rs and other organisations focusing on 3Rs e.g. https://3rc.org/. Should there be an advancement that allows for us to improve our protocols, in respect to the 3Rs, we will gladly do so.

24. Oral and dental regeneration and repair

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Oral and Dental, Stem cells, Repair, Regeneration, Diseases

Animal types	Life stages
Mice	neonate, juvenile, pregnant, embryo, adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand how oral and dental tissues maintain their integrity and function in health and when affected by common dental diseases such as caries and periodontitis, as well as their relationship with systemic diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Tooth loss is a global health problem representing a burden to society and the economy. It affects an individual's capacity for biting, chewing, smiling, speaking, and psychosocial wellbeing. Dental caries, periodontal disease, and genetic disorders are major causes of tooth loss. These diseases continue affecting the global population during life due to their chronic characteristics, particularly affecting older people. Additionally, current dental treatments are based on using inorganic materials and appliances, which are suboptimal and do not affect positively general health. Therefore, we will generate knowledge to develop methods to improve natural tissues healing during damage or disease, similar to what happens in humans, to create ways for treating oral diseases that can ultimately improve diseases in the whole body.

What outputs do you think you will see at the end of this project?

The project will deliver substantial academic outputs directly aligned with the core aims of understanding and treating oral diseases through regenerative medicine. The findings of this project could subsequently benefit clinical and economic domains.

Impact and Outputs: Within five years, this research aims to establish foundational methods for natural tissue regeneration, evidenced by preclinical models demonstrating enhanced repair of oral tissues and improved systemic health. Clinically, these outputs will lay the groundwork for trials in regenerative dental treatments, reducing reliance on inorganic materials and offering patients solutions that improve both oral and systemic health. The absence of such advancements would leave a significant gap in the approach to treating oral diseases, which currently overlook the regenerative potential of biological treatments.

Academic Impact and Outputs: Academically, this project will yield substantial contributions to the fields of dental regeneration and preventative medicine, including peer-reviewed publications, data on stem cell and microbiome dynamics in oral repair, and a comprehensive framework for future research into regenerative therapies. This work will expand the scientific community's understanding of oral health's role in systemic disease, supporting a robust foundation for further investigation. Without these findings, the link between oral and general health would remain inadequately explored, limiting advancements in preventive health strategies.

Future economic Impact and Outputs: By reducing the frequency and invasiveness of treatments needed for chronic dental conditions, this work will generate cost-effective strategies that could significantly alleviate healthcare burdens, particularly for services like the NHS. The absence of these advances would perpetuate the economic strain associated with traditional dental care, especially given the chronic nature of diseases such as periodontitis.

Targeted Beneficiaries: Initially the research team and the research community will benefit from this project's outputs. Short-term benefits will include new insights and models that guide clinical practice and inform future research, while long-term benefits may revolutionize oral disease management and its systemic health implications, impacting patients and clinical guidance.

Who or what will benefit from these outputs, and how?

The expected benefits from this project will be in 2 broad categories.

1. Restorative and regenerative dentistry with the aim to translate new treatments to human trials

2. Gum disease development and treatment, and how it impacts diseases in the whole body Tooth disease such as caries is a major drain on health resources worldwide. Although teeth can

naturally repair themselves, the damage caused by dental caries or fractures, is too extensive for this natural repair to be effective. This research will use genetic mouse models in which teeth are experimentally damaged to study the processes of natural repair. The eventual aim will be to develop ways of improving this repair.

Periodontitis is the major disease of teeth and the main cause of tooth loss. It is an inflammatory disease caused by bacterial infection that destroys the periodontal ligament leading to bone loss, during life. This project aims to develop a new generation of biological treatments for this disease to restore tissue loss and prevent bone destruction and tooth loss, during the lifespan of humans.

How will you look to maximise the outputs of this work?

The cross-disciplinary nature of this project will allow us to publish our findings in top international journals and successful completion of the project will represent the first example of studies where modulation of physiological mechanisms that control general health have been directly linked to preventing oral disease through one's lifespan.

All together the knowledge generated will benefit widely across the academic collaborators in the fields of cell biology, microbiology, stem cells, and preventative and regenerative medicine/dentistry field.

Species and numbers of animals expected to be used

- Mice: 1700

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Teeth are fundamental for mastication, and tooth loss due to diseases such as caries and periodontitis, and is a major healthcare burden. Rodents are prime model animal to study teeth and in particular, genetically-modified mice provide ways to study the molecular and cellular basis of these diseases and also reproduce the diseases so that novel treatments can be developed. Rodents have two types of teeth, 1) continually growing (incisors); 2) static similar to humans (molars). These two types of teeth, allow us to understand both the biology of how teeth repair, disease establishment, and to test new treatments to understand the potential of evolving the profession.

The proposed project uses wild type and genetically altered mouse models to study normal and abnormal oral tissues and disease to improve the basic understanding of the underlying mechanisms of these diseases. In addition, this project will aid development of new treatment therapies for these condition in all stages of life, from early adulthood all the way to geriatric stage of the animals.

Typically, what will be done to an animal used in your project

Tooth loss, Repair and Regeneration:

Tooth decay is a major burden on human health and the commonest non-infective disease. Current clinical treatment does not restore vital tooth tissue and new treatments are needed that are able to restore both hard and soft dental tissues following caries removal. Experimental damage to teeth to mimic caries is used to investigate the mechanisms of natural repair and regeneration.

We use this model to create experimental damage to tooth crowns and apex that can mimic dental caries, tooth fracture and pulp exposure. Molecules, metabolic modulators, and biomaterials can then be administered either locally or systemically to stimulate natural repair of the tooth hard tissues. Since this experimental damage does not include any infective agents that are found in human dental caries, additional agents such as bacterial products may be added to mimic the consequences of tooth pulp infection. This is done to approximate as much as possible the situation that happens in humans. The addition of bacterial products is also helpful to understand the influence of periodontal disease on tooth repair and regeneration. This model thus provides the experimental basis for the development of new biological approaches to stimulate natural repair and regenerative processes in the tooth that will form the basis of a new generation of dental treatments.

Additionally, the mouse incisor is an excellent model to understand stem cell biology. The incisor loop houses a niche of epithelial and mesenchymal stem cells that continuously give rise to the ever growing incisor. By understanding how the incisor stem cell niche works, in homeostasis and damage response, we further refine our knowledge of dental pulp stem cell activation and generate novel treatments based on stem cell activation in the molars, an organ that is more similar to human teeth. Additionally, we will use the incisor model to investigate the effect of ageing on this stem cell niche, and the concept of stem cell exhaustion. Given that the incisor continuously grows every 2 days following sectioning, we will investigate the timing of stem cell exhaustion following incisor sectioning, so that we can try and recover stem cell function in aged niches. This way, we can extrapolate the findings to aged molars, as they do not repair as a young molars. Therefore, potentially finding novel treatments for aged teeth.

Tooth loss is a consequence of extensive tooth decay and periodontal disease. Following a tooth loss event, the alveolar bone repair takes place. Current clinical treatment for tooth loss is the placement of a dental implant. If too much alveolar bone resorption takes place following tooth loss, restoration of the tooth with a dental implant is not possible. Moreover, periodontal disease affects the biology of healing, so patients with periodontal disease do not normally have implants. Therefore, experimental tooth extraction to mimic tooth loss is used investigate the mechanisms of natural repair and regeneration of the alveolar bone, in health and disease. We use this model to create experimental tooth loss that can mimic the loss of teeth and alveolar repair. Biomaterials and/or cells can then be used to study

how to optimise alveolar bone preservation and regeneration so that restoration of the dental function can take place.

The bone in the maxilla and the bone in the mandible, differ in mineral volume, with mandibles having more trabecular bone when compared to maxillas. The impact of this physiological change is not very well explored, therefore, in our model we will deploy bilateral maxillary and mandibular extractions (limiting ourselves to the first molars) to investigate the biology of these different bones, as well as the response to biomaterials, small molecules and pharmaceuticals. A bilateral approach (split mouth) helps us diminish animal number as we can deploy a split mouth approach.

Given the nature of mineral and soft tissue repair that tooth drilling and extractions needs, we will employ in vivo imaging, allowing us to time-lapse repair at different stages of tooth manipulation, and decrease animal numbers.

Periodontal disease and ageing:

Oral diseases are highly prevalent around the world and at the biological level it is natural for them to widely arise in the human species during their whole lifespan. Periodontal disease is the major cause of tooth loss and is directly associated with systemic diseases that arise in late adulthood to geriatric stages. Therefore, there is a need for novel interventions that prevent and/or cure oral diseases at a more fundamental level, targeting the biological processes underlying these diseases during the lifespan.

Mice do not naturally develop periodontal disease and so to develop new treatments, the disease has to be stimulated in the mouse oral cavity. Several different methods to mimic periodontal disease in mice have been developed and are widely used. No single method completely reproduces all the features of human periodontitis but each one reproduces certain specific features. In order to investigate news ways to treat the disease the different mouse models all need to be used.

Periodontal disease models involve:

1- colonization of the mouth with specific bacteria known to cause periodontitis in humans. This mimics the initial insult from which periodontitis begins.

2- administration of bacterial products to mimic aspects of periodontitis. Bacterial products are easier to use and more reproducible that intact bacteria, however they do not mimic all the effects of a bacterial infection.

3- using a ligature around the tooth periodontal ligament to stimulate immune responses including inflammation, that are induced in periodontitis. This method allows the changes in immune cell infiltration locally to be analysed in the absence of bacteria or bacterial products or secondary effects from other tissues.

4- bone loss is a natural process of aging. In the periodontium, the bone loss seen in geriatric mice without disease induction is equivalent to those of young mice that had periodontal disease induced. Understanding how this aging-related natural bone loss in health and disease affects the oral and systemic environments during the lifespan of the animal can help elucidate how to prevent oral and systemic diseases that are associated with periodontal disease.

These different disease models will be used to provide microbiome (bacteria) analysis and cells for gene expression analysis to reveal changes in dynamics of the tissue-microbiome as a result of the disease. using this model, we can also evaluate systemic markers or ageing related diseases, such as glucose levels, blood markers of inflammation, and cognitive signs of degeneration. All of which are directly associated with periodontal disease. These datasets will then inform the selection of signalling pathways and metabolic markers to be targeted to restore health and increase prevention of these affected diseases.

The delivery of agents that can activate cell proliferation and differentiation in the periodontal ligament will be investigated with these different models of the disease to determine their effectiveness as treatments and the relative impact of bacteria, their products and immune cell responses and infiltration. To track the response to treatment, and to decrease the animal number used in the experiments, we will employ in vivo imaging, allowing us to time-lapse the effect of periodontal disease progression.

Gene and metabolic modulation could offer a potential avenue for altering host regulatory genes that are an integral part of fighting against oral diseases development. Glucose metabolism modulation have been shown to affect levels of inflammation in the body, increasing longevity in vivo and in human trials. Moreover, the use of gene modulation techniques that target anti-inflammatory, metabolic, developmental, and healing pathways provides a new avenue of exploring and harnessing body’s natural mechanisms to regenerate oral tissues. For future translation to clinical trials agencies such as the MHRA need to be convinced that appropriate animal models have been used that reproduce all aspects of the disease.

What are the expected impacts and/or adverse effects for the animals during your project?

Breeding and maintenance of genetically altered animals/wild type animals: In general, animals produced under this protocol are not expected to exhibit any harmful phenotype.

Induction of genetic labelling for tracing: When the immune status of the animals might compromise health, they will be maintained in a barrier environment. Animals exhibiting any unexpected harmful phenotypes will be killed by a Schedule 1 method, or in the case of individual animals of particular scientific interest, advice will be sought from the vet.

Our preferred method of tamoxifen delivery is via either diet or oral gavage, however, when tamoxifen is administered via the intraperitoneal route, it may cause abdominal discomfort. We will limit the delivery of the substance to as few injections as needed to induce the desired effect..

Mutations are created that contain loxP sites to permit recombination following expression of cre recombinase. Cre lines that are used to generate mutations postnatally using tamoxifen administration may cause harmful effects to animal physiology.

Tooth regeneration and repair: The most likely adverse effect expected is the death resulting from anaesthetic or surgical complications. These are, however, uncommon (< 1%), and will be minimised by ensuring correct dosing of anaesthetics by accurate weighing, and by good maintenance of body temperature, e.g. by use of heated pads.

Clipping or drilling of a tooth, and tooth extraction may cause transient discomfort and may also lead to bleeding. Animals will be closely monitored after the procedure an if there are signs of pain analgesics will be administered. Bleeding is likely to cease without any treatment.

There is a chance of malocclusion following clipping and tooth extraction resulting in inadequate tooth wear during feeding. Mice will be monitored for loss of weight, dehydration and visible problems with the formation of the incisors.

With regards to administration of adjuncts to the protocol described above:

No adverse effects of agents administered systemically eg: BrdU and liposomes are anticipated at the dose rates to be used.

The possible adverse effects of agents administered locally into the tooth (step 3) eg. LPS , small molecules, proteins, at the doses rates to be used are locally related to dental pulp death. There is no data to show that they cause systemic adverse effects, when added to teeth.

Periodontal disease induction and ageing:

It is not anticipated that there will be any adverse reactions to the exposure of bacterial strains and bacterial products.

Human periodontal disease is a painless condition and the mandibular bone loss induced by some of the organisms in this study is likely to be well tolerated by the mice.

The procedure of ligature placement in this study is likely to cause symptoms such as pain, irritation, reduced appetite leading to weight loss and abdominal pains etc.

Ageing of mice to >12 months may result in consequential age-related adverse effects including tumours, arthritis, hair loss or stress due to single housing. This is anticipated to affect less than 10% of animals maintained on this protocol, and animals will not be maintained if they show evidence of any unexpected adverse effect that has more than a mild impact on their welfare. Internal tumours may be manifested as abdominal swelling.

In rare cases, long term socially housed mice may experience aberrant grooming or aggressive behaviour from litter mates, which may necessitate single housing.

Any animals displaying evidence of an age-related issue will be closely monitored. Tumours will be detected by routine monitoring and will be checked for pain, for size, whether they are impeding movement, and how loose or attached they are, and if necessary, NVS advice will be sought.

Abdominal swelling will be monitored daily

Aged animals displaying any evidence of arthritis, such as reduced mobility/limb stiffness or signs of joint pain, will be treated with the appropriate NSAIDs in consultation with the NVS. We developed a 'traffic light' system to monitor the health of the animals that will be aged, which will help us maintain them with humane endpoints.

To prevent stress due to single housing, animals will be socially housed where possible. If this is not possible, either due to specific study requirements, or due to the potential for aggressive behaviours or over grooming, animals will be monitored for signs of stress, and may be provided with additional environmental enrichment. In specific cases of interest, or where an acute problem or injury is detected due to over grooming or mouse fighting, affected animals will be separated, and may be treated under advice from the NVS and/or NACWO.

With regards to administration of adjuncts and examinations within the protocol described above:

Blood sampling: Some local redness or swelling may occur at sites of the blood sampling (tail). Limits for blood sampling (maximum 10% circulating blood volume in 24 hours and 15% in any 28- day period) mean that hypovolaemia and anaemia is not expected to occur.

Medication: In case of oral administration of the agents/molecules given for the lifetime of the animal, the animals will be monitored, and procedure stopped if any signs of harmful effects or discomfort is observed. After administration of agents that lower blood glucose levels, e.g. metformin, glucose will be monitored and in the rare occasion that blood glucose levels fall below 2mM, glucose will be administered.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The first protocol will fall under the mild category (40%), and the last two protocols of this project will fall under the moderate category (60%).

What will happen to animals used in this project?

- Used in other projects
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

At present the study of tooth and oral regeneration and repair necessitates the use of animals both to provide a source of tissues and cells, to study the effects of genetic modifications and to investigate cell differentiation using lineage tracing.

We need to use animal models to understand how oral and dental tissues maintain their integrity and function in health and when affected by common dental diseases such as caries and periodontitis, as well as their relationship with systemic diseases. The tooth is complex organ composed of the hardest minerals the body can produce, living cells inside of this shell of mineral, and ligaments attaching it to bone. These diseases that affect teeth

are initiated by bacterial infection and the body response progresses the diseases, exacerbating systemic diseases. These processes and the dental organ are so complex that currently no in vitro system is able to replicate this. Therefore, we will gather information on how these interactions take place in living animals so that we can use this information to develop methods to improve natural tissues healing during damage or disease and to create ways for treating oral diseases that can ultimately improve diseases in the whole body.

Current alternatives include in vitro 3D gingival models, ex vivo tissue culture, and engineered tissues. Unfortunately, these models only work to understand their own separate and isolated tissue repair biology, disregarding the impact of the joint physiology of dental repair that comprises of mineral tissue, dental pulp, gingiva, cementum, bone and periodontal ligament acting together to reconstruct the tissues. Therefore, in vivo models are essential to evolve periodontal disease management.

Which non-animal alternatives did you consider for use in this project?

I have employed an online search, according to the Frame.org suggestions on alternatives to the models I am proposing in this licence.

Tooth manipulation:

For the tooth manipulation protocol, it involves: Tooth repair and tooth extraction.

The following search words were used on Pubmed: “Tooth repair in vitro”, “dental pulp repair in vitro”, “tooth extraction in vitro”, “dental socket healing in vitro”, “alveolar bone repair in vitro”

One article was found for dental pulp repair in vitro (https://doi.org/10.1177/00220345960750120901) However the models described in this publication disregard the immune cells present in the animal, which my group has shown that has direct impact on regulating dental pulp/tooth repair in vivo (https://doi.org/10.1038/s41598-020-77161-4)

No publications describing models for tooth extraction alternatives were found. Therefore, there is no model that can alternatively replicate in vivo models proposed in the tooth manipulation protocol.

Periodontitis and ageing:

The periodontitis and ageing protocol involve induction of periodontal disease via different methods and ageing of the animal for natural ageing-related periodontal bone loss.

The following search words were used on Pubmed: “Periodontitis induction in vitro”, “alternatives to gingival inflammation in vivo”, “3D periodontal models in vitro”

Two articles were found with the “3D periodontal models in vitro” search words: Gingival 3D models: https://pubmed.ncbi.nlm.nih.gov/37938480/

Periodontal ligament 3D models: https://pubmed.ncbi.nlm.nih.gov/25526626/

Additionally, we hold knowledge to undertake in vitro models to understand the impact of bacterial/bacterial products on primary culture of bilayer oral mucosa (https://doi.org/10.1038/bjc.2011.403).

Nevertheless, unfortunately, these models only work to understand their own separate and isolated tissue repair biology, disregarding the impact of the joint physiology of periodontal repair that comprises of gingiva, cementum, bone and periodontal ligament acting together to reconstruct the tissues. Therefore, in vivo models are essential to evolve periodontal disease management.

Why were they not suitable?

In vitro models cannot replicate the complexity of microbiome and organ model repair model during physiological function as described in the previous answer.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

1000 for breeding and maintenance of genetically altered animals 350 for tooth loss, regeneration, and repair

350 for periodontal disease

Our experience with the protocols proposed in this licence, both using wild type and genetically- modified animals, led to quantity estimation of the quantity of animals that will be used in this project, taking into account the variation in disease progression and healing between individual animals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We based our estimate on the required number of breeding animals required to obtain the required number of experimental animals for the procedures we have planned. For the experimental animals we consulted with our statistician colleagues to get a statistical estimation of minimum numbers of mice required to achieve a meaningful result (statistical significance). This is based on our previous studies using these models of tooth damage/manipulation and periodontal disease taking into account the variation in disease progression and healing between individual animals.

For example we previously used 4 mice/group to investigate differences of repair after tooth drilling, and 3 mice/group to investigate cell changes when the mouse incisor was sectioned. For tooth extraction, we used 5 mice/group to investigate differences of how materials affect bone repair. And for periodontal disease and ageing, we used 6 mice/group to investigate differences of treatment in periodontal disease.

Additionally, the NC3Rs Experimental Design Assistance will be consulted for use of systemic pharmaceutical use.

We also aim to reduce the number of animals is that all tissues will made available to collaborators. This will reduce the number of animals used, as multiple tissues and organs will be study from the same animal.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Our previous discoveries have highlighted methodological optimisation of animal use to understand common cell processes during oral tissue homeostasis and during injury. Our experience with the protocols proposed in this licence, both using wild type and genetically-modified animals, led to quantity estimation of the quantity of animals that will be used in this project to study cells in teeth, and in other craniofacial tissues, bone, cartilage etc.

With the use of in vivo imaging, we will be able to decrease drastically the use of animal numbers, for example:

For analysis of tooth repair, I would ideally understand the mineral content in the injury site at 1,2,4, and 6 weeks after damage, leading to the need of at least a total of 32 animals in case we have one test and one control group (n=4 per treatment per time point). With in vivo imaging, we can decrease this number to 12 animals (n=6 per treatment – only 2 groups), a 60% reduction in animal use.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

In this project, we will use genetically modified mouse models to study oral and dental tissues' integrity and repair mechanisms. Specifically, we will use oral gavage for tamoxifen induction, as this method is less invasive and minimizes discomfort compared to intraperitoneal injections. Post-surgical care will include the administration of analgesics to alleviate any pain and ensure the well-being of the animals.

To refine our surgical methods, we will ensure that the sizes of the injuries are appropriate and necessary for the study, avoiding any excessive or unnecessary damage. We will employ advanced intra-oral surgical techniques that are less invasive, such as using burrs to section the incisors, instead of the traditional tooth clipping, therefore promoting a more predictable recovery. Additionally, a bilateral molar extraction approach helps us diminish animal numbers as we can deploy a split-mouth approach. Animals will be provided with heat pads during their recovery phase to maintain their body temperature and enhance comfort post-surgery.

Our approach includes using in vivo imaging techniques, such as micro-CT , to monitor tissue repair and disease progression over time. This allows us to minimize the number of animals used in the study by enabling longitudinal studies on the same subjects, thereby reducing the overall impact on the animal population.

Overall, our methods are designed to minimize pain, suffering, distress, and lasting harm to the animals, ensuring that their welfare is a top priority while still achieving the scientific objectives of the project.

Additionally, some animals will be allowed to grow old to study the interaction of oral and systemic diseases in the ageing animal. In order to holistically investigate the mice, we will have to draw blood, and undertake cognitive/memory examinations.

To refine the model for studying the interaction of oral and systemic diseases in aging animals, we will implement several measures to minimize distress and enhance animal welfare. Blood draws will be conducted using minimally invasive techniques, such as tail vein sampling, with local anaesthetics applied to reduce discomfort. Cognitive and memory examinations will be designed to be non-stressful and incorporate natural behaviours, such as maze navigation and object recognition tasks. Additionally, aged animals will be provided with enriched environments to promote physical and mental well-being, and closely monitored for any signs of distress or discomfort, ensuring timely interventions as needed.

Why can’t you use animals that are less sentient?

Mice are the main animal model used since they are the mammalian species most appropriate for studies based on genetic analysis. Their small size together with the extensive international resources of genetic-modifications make them the only species for the proposed research. The common other developmental animal models used such as avians or fish do not have many of the mammalian- specific orofacial organs such as teeth, tongue, salivary glands etc.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Most of our procedures are mild, which produce minimal and transient discomfort to the animal. The remainder of our procedures are mild/moderate and all stress and pain is appropriately monitored and minimized throughout. Stress is minimized pre-operatively by not keeping animals waiting in the same room as a procedure is carried out and with minimal handling. Perioperative analgesics will be used to minimize pain and discomfort, and all animals will be given a warm area to recover. All animals will be closely monitored post-operative for any signs of discomfort and will be treated appropriately.

Tooth clipping is a widely used technique to assess incisor growth, however, clipping may leave the tooth with chips due to irregular cuts. Therefore, animals that will undergo incisor sectioning, will have the procedure done with a drill, so that the sectioning of the tooth is uniform, without causing chips.

Ageing animals will be carefully monitored by staff trained to work with ageing animals. Group sizes in ageing experiments will be increased to accommodate for loss of animals and to avoid single housing due to animal losses due to old age. Longer drinking spouts will be used, and animals will be monitored for adverse effects.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

PREPARE guidelines; The LASA surgical guidelines; The ARRIVE guidelines; Morton et al. 2001 for the administration of substances; and S Parasuraman et al. 2010 for mouse blood sampling.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly check information on NC3Rs website and attempt to acquire grants to develop 3Rs methodology.

25. Development and function of blood and immune cells

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

T-cell, thymus, haematopoiesis, immunity, blood cells

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to investigate the molecular mechanisms that control the development and function of cells of the immune system and other blood cells. All blood cells including cells of the immune system are produced from stem cells through a process called haematopoiesis. We will investigate the way in which these developing cells interact with their environment during haematopoiesis and the way in which their environment regulates their development, fate and function.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Immune cells are essential to protect us from infectious disease and cancer, but if their development or function goes wrong, they can lead to many diseases, such as blood cancers, autoimmunity and immune-deficiency. It is therefore very important to understand the molecular mechanisms that regulate the development and function of blood and immune cells, in order to develop strategies to enhance immunity against infectious

disease and cancer, and to develop strategies to treat and prevent diseases of dysregulated immunity and haematopoiesis, such as blood cancers and autoimmunity.

What outputs do you think you will see at the end of this project?

This project will lead to new information about how immune and blood cells interact with their tissue environment and how these influences affect their development and function.

This information will be published in peer-reviewed open-access journals and presented at scientific meetings.

Data generated from this project will also be made publicly available on publication through public data repositories.

Who or what will benefit from these outputs, and how?

Academic beneficiaries of this project will be other groups working in the fields of immune and blood cell development and function, blood cancers, autoimmunity and immunity.

We aim to publish data from this project as soon as possible after its collection, and maximally within 3 years of its publication. Large electronic datasets (for example, next generation sequencing datasets) will also be made publicly available through deposition in public data repositories at the time of publication, or within three years of data generation. Data from the project may also be included in PhD theses of students, and these PhD theses will be made publicly available by our institution.

How will you look to maximise the outputs of this work?

In addition to the publication of successful approaches, we will make negative data available ("unsuccessful approaches") through public data repositories, scientific meetings, PhD theses and publication in open-access journals.

Species and numbers of animals expected to be used

- Mice: 12000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using mice as a model animal for mammalian (including human) Immunology and haematopoiesis. Haematopoiesis is the process by which all blood cells are produced throughout the life of an individual.

Use of animals is essential, because we study dynamic physiological systems (immunity and haematopoiesis) that cannot be investigated in isolation, in which there is homeostatic control, and multiple cell types and tissues interact through time. Mice are most appropriate species because relevant genetically modified mice are already available.

Mice have long been used as a model to investigate immunology and haematopoiesis and our findings will be interpretable within the context of this wide body of literature.

Mice are an excellent model system for investigation of immunology of other mammals and nearly all our understanding of mammalian immunology is based on findings from mice studies.

Most of our studies will be carried out on young adult mice. Where possible, we will carry out ex vivo analysis, or use animals as source of tissues only, rather than experimenting on live mice. However, to investigate the immune response (response to vaccines and tumours, atopic skin and lung inflammation) use of live mice is essential, as we will immunise mice to investigate the immune response through time.

The immune system and the development of immune cells deteriorates with age, so in some experiments to investigate the impact of ageing on immune development and function, we will carry out experiments on old mice (>12 months).

Diet and obesity also affect the immune response and the development and function of blood cells. Therefore in some experiments we may feed mice a high calorie diet to induce obesity and investigate its effect on immunity and haematopoiesis. As induction of obesity will take around 3 months, these experiments will be carried out on mice of approximately 6 months of age.

We will breed genetically modified mice to obtain tissues in which a gene has been inactivated/modified. To obtain foetal tissues to investigate immune cell development, we will mate mice to produce embryos and the embryos will be sacrificed to provide tissues for in vitro experiments.

Typically, what will be done to an animal used in your project?

The majority of mice bred on this project will be genetically modified mice that will be bred as a source of tissues only.

In some experiments mice will be maintained on a high calorie diet to induce weight-gain and obesity.

In some experiments we will immunise mice to investigate their immune response. These experiments will typically involve injecting mice. This may be carried out on several occasions at about 4 week intervals.

In some experiments, mice will be immunised by putting a substance on their skin, or by inhaling a substance into their lungs. This may be carried out several times, usually within a two week period.

In some experiments mice will be given a non-lethal dose of irradiation. In some experiments mice will be kept to old age (more than one year).

What are the expected impacts and/or adverse effects for the animals during your project?

For experiments in which mice are injected with a substance or cells we expect no adverse effects apart from transient discomfort at the site of injection.

For experiments in which mice are immunised by treatment of skin with a substance, some redness and swelling may be observed at the site of treatment. The maximum duration of this effect is expected to be two weeks.

For experiments in which mice are immunised by inhalation of a substance into the lungs, we do not expect adverse effects.

For experiments in which mice are fed a high calorie diet, we expect no discomfort to the animals, but we expect the animals to show significant weight gain.

In some experiments mice will be aged for more than one year before immunisation. Older mice have higher risk of developing tumours, or problems with movement and sight. The maximum duration for which we will keep mice that have been aged to more than one year is 6 months.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

For most experiments the severity will be mild or sub-threshold (breeding of genetically modified mice as source of tissues, immunisations with vaccines or cells, non-lethal radiation, maintenance on high- calorie diet).

In experiments where mice are immunised by treatment of skin, maximum severity is moderate, but expected severity is mild.

In experiments where mice are kept to old age (> 1 year), the maximum severity is moderate, but expected severity is mild.

What will happen to animals used in this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We are using mice as a model animal for mammalian (including human) Immunology and haematopoiesis.

Use of animals is essential, because we study dynamic physiological systems (immunity and haematopoiesis) that cannot be investigated in isolation, in which there is homeostatic control, and multiple cell types and tissues interact through time. Mice are most appropriate species because relevant genetically modified mice are already available.

Which non-animal alternatives did you consider for use in this project?

Where possible we will use in vitro culture systems to investigate haematopoiesis and lymphocyte development, and animals will be used as a source of cells or tissues only. For example, we will use Foetal thymus organ culture to investigate T-cell development in the thymus.

To investigate immune cell function, we can activate cells from genetically modified mice in culture systems and investigate the impact of the mutation on their function.

Why were they not suitable?

When we want to investigate the fate of newly developed T-cells when they leave the thymus and enter the blood system, spleen or lymph nodes, we will be unable to do so using Foetal thymus organ culture, and we will need to experiment on live mice.

To investigate immune cell function, we again need to immunise live mice, as cells of the immune system migrate in the body to the site of immunisation or to the lymph nodes so this process cannot be investigated using culture systems. In our studies we will investigate how different routes of immunisation (eg. skin, lung, injection) affect the immune response, and this cannot be done without immunising live mice.

To investigate how haematopoiesis recovers after irradiation, we cannot use in vitro culture systems because we want to investigate the homeostatic mechanisms that feed back to tell the animal to make more blood cells, and this can only be done in live animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals to be used during the project has been estimated to take into account the number of animals required to breed and maintain colonies of the required genotype and to generate animals to be used in experiments, bearing in mind that not all animals that are bred will be of required genotype, and that experiments must be repeated at least twice to avoid spurious results.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To reduce the number of animals required we have used NC3R's Experimental Design Assistant.

Experiments are planned according to NC3R guidelines so we make sure to use both genders of animals.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We carry out pilot experiments to determine appropriate group size, based on power calculations.

When genetically modified mice are sacrificed we use multiple tissues in different projects (sharing of tissue) both within our own group and with other labs at our institution.

We plan breeding carefully to achieve efficient breeding.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We have chosen to use mice because relevant genetically modified mice are available and technology to generate genetically altered mice is well-developed. Mice have long been used as a model to investigate immunology and haematopoiesis and our research findings will be interpretable within the context of this wide body of literature. Mice are an excellent model for human immunology and haematopoiesis (production of blood cells), and nearly all our understanding of human immunology and haematopoiesis is based on findings from mice studies.

The use of genetically altered mice allows us to test directly the impact of the factor or signalling pathway we are investigating on the immune response.

We may maintain aged genetically altered animals (>1 year) in order to investigate the influence of time on the impact of their genetic alteration on their immune organs and we may maintain some mice on a high-fat/high-sugar diet in order to investigate the impact of obesity on immune cell development and function.

Most of the animals used in this project will be bred and euthanized humanely in order to provide genetically modified tissues and cells for our laboratory experiments.

In order to investigate the immune response, some animals used in this project will be immunised. Immunisation would involve either an injection, or application of a substance to the skin, or making the mouse inhale a substance placed as a drop on its nose. In most cases, immunisation will not cause any pain, distress, suffering or lasting harm to animals, and it is important to enable us to investigate the immune response. To avoid suffering, we will monitor animals for any signs of distress and euthanize if they are deemed to be suffering.

In order to investigate how red blood cells are made during recovery from anaemia, mild anaemia will be induced in some mice, either by injection or by mild irradiation. This degree of anaemia will not cause suffering to the mice.

In some experiments surgery will be performed to transplant a piece of tissue under the skin or a small piece of tissue will be inserted under the skin by injection. This is not expected to cause suffering because anaesthesia and pain management will be used, and mice will be carefully monitored after surgery.

Why can’t you use animals that are less sentient?

We have chosen to use mice and not less sentient animals because mice have long been used as a model to investigate immunology and haematopoiesis and our research findings will be interpretable within the context of this wide body of literature. Mice are an excellent model for human immunology and haematopoiesis (production of blood cells), and nearly all our understanding of human immunology and haematopoiesis is based on findings from mice studies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be raised and housed under strictly controlled conditions and all scientific procedures are carried out as humanely as possible. The impact of an experiment on animal welfare will be carefully considered from the planning phase right through to the end of the procedure. Research will be designed to minimise the use of invasive or distressing procedures where possible, and anaesthetic will always be used where appropriate.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow guidance provided by the

The UK National Centre for the Replacement, Refinement and Reduction in Animals in Research (NC3Rs). https://www.nc3rs.org.uk

Understanding Animal Research .https://www.understandinganimalresearch.org.uk Universities Federation for Animal Welfare. https://www.ufaw.org.uk

Laboratory Animal Science Association (LASA) https://www.lasa.co.uk

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed about advances in the 3Rs through the support of our University's Central Biological Services, who provide professional advice about Replacement, Reduction and Refinement in research using mice, through their newsletters and meetings. We will also discuss innovations that support advances in the 3Rs with our named veterinary surgeon and local professional animal house staff.

To reduce animal experimentation, our experiments will always be designed to use as few animals as possible to answer the scientific question being investigated and we will record as much data as possible from each experiment so that the experiments will not need to be repeated as often. We will also re-analyse data from past experiments using modern techniques to avoid using animals again.

We will keep abreast of scientific literature and innovations in order to strive to replace animal research with alternative methods where possible. These will include computer modelling, tissue culture and statistical studies.

26. Generation and differentiation of blood and the cardiovascular system

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

stem cells, haematopoiesis, cancers, marrow failure, zebrafish

Animal types	Life stages
Zebra fish (Danio rerio)	embryo, juvenile, adult, neonate, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Our overall aim is to understand how the blood and circulatory systems are made, and how their function is impaired or corrupted in disease such as cardiovascular disorders and blood cancers.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Each organ in our bodies is made of different types of cells that are made from early ‘precursors’ during embryonic development in the womb. This includes the cells that make up our blood and circulatory systems. To understand how these are made and how they go wrong in disease, we need to identify and assign specific functions to the genes and proteins that are present in these tissues. A more profound understanding of the mechanisms controlling tissue-specific gene expression will shed crucial light on congenital and acquired human diseases affecting circulation or blood formation (for example, blood cancers), and it will enable scientists to find new ways to modify or harness the underlying biology to prevent or delay disease onset or progression. This work

will also provide a framework that other researchers can make use of to develop better tools for understanding these diseases or to design therapeutic strategies to combat them.

What outputs do you think you will see at the end of this project?

In this project we are investigating the development, maintenance and survival of blood, blood stem cells and the circulatory system. We list here the expected outputs from each of our main objectives (Primary purpose: advance science; secondary: disease control):

1. Identifying the ways in which cells in our organism communicate with each other and coordinate their functions to form blood vessels and blood and their stem cells.

2. Identifying adult stem cells and elucidating their programming will enable a better understanding of their normal and defective behaviour and how it may be controlled. We will directly compare normal blood stem cells with those found in marrow failure or leukaemia-predisposition models in zebrafish to identify novel targets that could be used to inform patient stratification or to design new therapeutic approaches.

3. Identifying ways in which normal and diseased cells interact with each other to contribute to disease onset or progression, with a view to target these interactions for patient benefit.

Who or what will benefit from these outputs, and how?

In this project we are investigating the principles of haematopoietic stem cell maintenance, differentiation and survival driven by transcription factors or enhancer elements in the genome. In the short term, this will be of great interest to other researchers in the field of blood formation (haematopoiesis) and stem cell biology, and any new discoveries will be shared by communications at conferences and publications in peer-reviewed journals.

Loss of function in key haematopoietic regulators in humans leads to haematopoietic disorders that may lead to leukaemia. Therefore, in the medium term, clinicians and clinical geneticists with interests in normal haematopoiesis and blood disorders leading to leukaemias will be especially interested in our research as it may have a more immediate impact on the genetic screening routinely used in the clinic and potentially provide platforms for testing of novel therapeutical modalities via genetic or chemical screening, for example. Our project will be of great interest to haematology researchers, geneticists and cancer biologists.

While our project is fundamental discovery science, the knowledge generated will be crucial for our understanding of the molecular mechanism underlying haematopoietic disorders in humans and provide insights into onset of disease and the transition from normal haematopoiesis to leukaemia. The beneficiaries of this research include patients suffering from haematopoietic disorders, cancer patients suffering from myelodysplastic syndrome (MDS) or leukaemia and health professionals. These are long-term benefits to these stakeholders that will likely only be felt beyond completion of this project.

How will you look to maximise the outputs of this work?

The discoveries we make during the course of this project will be presented at national and international conferences and published in peer-reviewed journals as appropriate. This will help to disseminate new knowledge but also to encourage new collaborations that might

result in a new body of knowledge that is more than the sum of its parts. An important part of this exercise is that it creates a forum for exchange of successful and unsuccessful approaches that will inform research efforts by others in the research community. We routinely publish primary research as well as protocols that we feel are useful to the research community, always as Open Access to ensure this knowledge is disseminated as widely as possible and without barriers. Any data that is not used for specific publications will be made publicly available (after a suitable period of embargo) in local or international repositories (PubMed Central, Figshare, etc) to ensure maximum exposure and usage of the data generated.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 14400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Zebrafish are genetically very similar to humans; the formation of blood and blood vessels is a well conserved process between zebrafish and human. Advances in the genome sequencing of zebrafish have enabled the use of cutting-edge techniques to manipulate the zebrafish genome, which we can make use of to study many signalling pathways and disease-related genes during embryonic and early larval stages. Very early signals are not easily studied in mammalian embryos in utero and the number of embryos available for study is limited. By contrast, zebrafish can generate large numbers of embryos by natural mating and the embryos are accessible for these studies.

The use of zebrafish avoids the use of mammalian models and has the advantage that the key characteristics of blood stem cells can be observed within a small time window in zebrafish embryonic and larval development. Therefore, most of our research will be conducted using larval forms of the zebrafish up to 5 days post-fertilization (dpf), before the commencement of free feeding when they become protected under the Animal Act 1986.

The advent of single cell technologies has also enabled us to undertake more precise studies of the blood formation process in adult animals, and how it may be affected in disease. In some cases, to more closely represent adult cell characteristics, we may need to study blood cell formation and function in both larval stages and in ageing adult animals. These are anteicipated to be the minority of the cases.

Typically, what will be done to an animal used in your project?

Typically the animals will undergo fin clipping, either as larvae or as adults, or skin swabbing as adults to identify the ones with the required genetic mutations. When fin clipping, a very small biopsy is taken from the tail fin under anaesthesia and analgesia as agreed with the veterinary surgeon and will cause only transient discomfort and no lasting harm. Both the larvae and adult zebrafish have an amazing regeneration capacity and the fin will completely regenerate in a few days. Most adult zebrafish in this protocol will be used for natural matings to generate larvae for analysis.

The genes involved in leukemia are normally expressed throughout development but when that expression is perturbed they are likely to cause disease in adults. We wish to determine how cancer- causing genetic mutations impact on normal blood stem cell lineage specification and differentiation in the adult. In these cases, animals will typically undergo fin clipping under anaesthesia/analgesia once and allowed to grow up to 24 months of age to study disease onset and progression where relevant. As our interest lies in the early biological events of disease progression, animals will usually be humanely killed if they show any major departure from their usual state of health. Post confirmation of death, biopsies may be taken for further analysis.

Where a gene of interest, or a combination of genes or pathways of interest are likely to play a crucial role later in development (post free-feeding), transgenic or mutant embryos may be injected with materials (e.g. DNA, RNA, CRISPR/Cas9), immersed in small molecule inhibitors or transiently exposed to higher temperatures (up to 37°C) that enable activation or inactivation of these additional genes or pathways. Embryos will then be allowed to grow past 5dpf and juveniles (up to 31dpf) may be immobilized under terminal anaesthesia in order to acquire a high resolution image with more specialized microscopes such as for example spinning disc or laser scanning microscopes. In these experiments, animals may undergo anaesthesia/analgesia once (before 5dpf) for genotyping and then undergo terminal anaesthesia for imaging up to 31dpf.

In order to understand how vasculature and the blood cell components in the embryo responds to instructions to generate new tumour-induced vessels, transgenic animals may be injected with tumour cells/organoids and grown up to 31dpf for imaging under terminal anaesthesia as described above.

In some occasions we may need to generate new genetically altered animals to help us understand how a particular gene or regulatory element contributes to normal or perturbed blood formation.

Typically, embryos will be microinjected with a material that causes alterations in the sequence or function of a particular gene of interest. Embryos will then be allowed to develop to adulthood and mated to wildtype to assess transmission of the required genetic modification. Occasionally, they may undergo fin clipping or imaging under anaesthesia/analgesia once to help identify the appropriate animals for breeding and line maintenance.

To understand how certain blood cell populations contribute to normal and abnormal blood formation, we will use a strain of fish that has a defect in this cell population as a host and transplant in donor blood stem cells that carry a ‘label’ (for example a fluorescent tag). This allows these donor cells to be tracked within the host fish over time. To achieve this, we will obtain cells from the donor fish either from the kidneys (obtained post-mortem) or via embryos. These cells will be injected into the host fish at embryo stages and then the host may be maintained up into adulthood. The host may be imaged under anaesthesia to monitor engraftment on a maximum of 5 occasions.

What are the expected impacts and/or adverse effects for the animals during your project?

Typically, animals in this project will be used for breeding and generating embryos and larvae up to 5dpf for analysis and thus the main source of adverse effects is likely to be from the use of anaesthetic prior to genotyping or from the genotyping itself. These effects

are temporary and animals are expected to return to normal swimming and behaviour within 30 minutes. If not, then they will be humanely killed.

In very rare cases animals may develop infections due to fin clipping or damage to scales due to loss of mucous surface from swabbing. If this occurs then animals will display abnormal behaviours and swimming in which case they will be humanely killed.

Adverse effects from the genetic modification - Genetically altered animals may display a harmful phenotype due to the genetic modification that may include redness in the skin and tissue swelling that may be indicative of impaired blood stem cell function. These should not affect their swimming or feeding behaviour but are indicative of an underlying phenotype. In these cases, animals will be humanely killed.

Adverse effects from the transplantation experiment – Host animals undergoing transplantation of donor cells will receive these during early embryonic stages. Engraftment of the donor cells may fail in around 5-10% of animals. In this situation, the host will grow at a slower rate than equivalent animals; these animals will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Sub-threshold – 55%
- Mild – 40%
- Moderate – 5%

What will happen to animals used in this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This project aims to study the formation and differentiation of blood stem cells, their journey between haematopoietic niches and their behaviour in these niches and in response to challenges (e.g. impaired stem cell function or solid cancers that hijack blood and endothelial cells). This requires a complex organism with an intact whole body system. There is no viable alternative available that faithfully mimics these process in non-animal models and thus we require the use of animals. However, in most cases we can address the objectives of this study by using larvae that are at a lower level of sentience (i.e up to 5 dpf), after which they become protected by law. Thus, while still requiring animals, where possible the majority of our aims will be achieved by using larvae instead of adults.

Which non-animal alternatives did you consider for use in this project?

We have considered using embryonic stem cells in culture for this project. However, in order to study tissue differentiation during embryonic development and their normal activities in the adult, or the interactions they show in complex tissues, it is essential to determine what happens in a developing embryo and how this translates into the adult. Cells in culture are not suitable for these types of studies. Where appropriate, publicly available data (from our lab and others) can inform on our research direction, for example by providing further inclusion/exclusion criteria for animal studies - this alternative in turn will help to direct the research efforts towards non-animal alternatives as appropriate. For example, we regularly use publicly available single cell transcriptional profiling data as a means to select genes for further study. In addition, we are already making use of alternative approaches to assess the molecular roles of proteins of interest (e.g. DNA damage assays) using in vitro cell culture assays, thus replacing the use of zebrafish to perform these experiments.

Why were they not suitable?

The developmental processes that we are studying are a constantly evolving system that requires complex temporal and spatial regulation to give rise to the blood and cardiovascular systems. Many cues directing these processes are received as cells interact with one another and migrate throughout the animal or particular organ/tissue and change with time and between different tissues. Thus these processes cannot be modelled in cell culture or in isolated tissues. However, new information arising from publicly available data or in vitro systems such as embryonic stem cells is incorporated in our strategy to narrow down potential targets of interest for animal studies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The animals in this project will be used for breeding of embryos for analysis before 5dpf, juveniles for imaging before 31dpf and adults for analysis of haematopoietic phenotypes up to 24mpf.

The guiding principle used for this estimation is to use the minimum number of animals that enables breeding and maintenance of specific strains and enough animals for experimental purposes. The number of animals was therefore estimated based on previous experience, overall usage during the course of my previous license and on in-house and published data that efficiently uses animals for research.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Because zebrafish develop externally, there is no requirement to sacrifice the mother (like in rodent models); rather embryos and larvae are obtained by natural matings. To

maximise the scientific productivity from single spawnings, group members routinely share embryos and larvae whenever possible. We also routinely perform various power calculations to ensure we produce the correct number of animals for statistical robustness. We will also reduce inter-tank variability by grouping animals of the same genotypes from different tanks where possible. For example, if 10 animals are required for a certain objective, we will take animals from different tanks rather than from just one.

Power calculations are performed using the EDA tool (NC3Rs website, allows t-test power calculations) or GPower (performs ANOVA power analysis and others not available with the EDA tool).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will continuously review our usage of the available fish strains and will categorize them into classes depending on their usage i.e high, medium, low and will accordingly reduce the number of fish we keep per line. Where we hold strains of scientific importance for the rest of the scientific community, then we will export it for long term storage at the European Zebrafish Resource Center (EZRC) . In addition, the animal facility provides an aquatic management software that allows the tracking of fish usage, including monthly reports and querying numbers of specific strains. We will also perform post- hoc power calculations to ensure the experiments are powered properly and perform pilot studies where appropriate.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

In most cases, we use zebrafish at early embryonic stages (up to 5dpf) and prior to inclusion under the Animals (Scientific Procedures) Act 1986. Fish embryos are transparent and easily obtained in large numbers by natural mating and the mother will be able to produce eggs on subsequent occasions. The use of such animal models from the lower vertebrates is a significantly less severe scenario from the animal's point of view since obtaining embryos does not imply surgical invasion of the mother with subsequent death as in mice. Thus, most of the analysis of gene function and cellular behaviour can be done non-invasively and ex-utero. Having the possibility to grow large numbers of embryos mean we can robustly generate enough embryos to grow to adulthood for further analysis of cell numbers, morphology or behaviour.

Our transplantation strategy makes use of genetically altered animals as recipients of transplants rather than inducing marrow depletion by conventional irradiation or chemical induction methods. This strategy avoids the potential damaging consequences for tissues other than the kidney marrow, such as the intestine.

This represents a refinement compared to inducing marrow depletion by conventional irradiation, a technique that shows low engraftment and high mortality rates in zebrafish (~70% based on published data). In addition, where we have a choice of host strains for the transplant work, we will first pilot the study in the strain that develops the least adverse effects. Only if this pilot fails will we then explore using a strain with more pronounced adverse effects.

Why can't you use animals that are less sentient?

The majority of the work is being done in zebrafish embryo and larvae at immature life stages (i.e. prior to free-feeding that occurs from 5dpf). This is a refinement that takes into account that at these stages they are less sentient than the adult zebrafish. The use of terminally anaesthetized animals is not compatible with the study of developmental events that take place over longer time scales (days) than would be safe to use or maintain anaesthesia. On the other hand, current in vitro alternatives are not yet proven to truly mimic normal development or disease progression in a complex multicellular organism. Although less sentient animals (e.g. Drosophila) are useful to investigate other aspects of the haematopoietic process, they are less complex and evolutionarily more distant from human and thus not appropriate for our project. For example, zebrafish have successfully been used to model disease progression like the development of solid tumours (e.g. melanomas), bone marrow failure and leukaemias and have provided a better understanding of these disease states. Thus zebrafish is most appropriate model to use that is considered less sentient than alternative mammalian models like mouse or rat.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Wherever possible, we aim to genotype animals by taking a small biopsy from the tip of the caudal fin before free-feeding; larval stage embryos are not as sentient as adults and their pain perception is not fully developed. In some cases, we will need to genotype adult animals. This will be achieved by skin swabbing or by collecting biopsies from adult tail fins. By contrast with current practice, which removes ~1/3 of the fin, our protocol uses instead a small biopsy that we estimate to be ~1/10 of the fin tissue. This protocol includes both anesthesia and analgesia to minimize any potential discomfort during and after the procedure. In our experience, >98% of the animals suffer only transient discomfort from the anaesthesia and rapidly return to normal behavior after fin clipping. However, we are reviewing this with the vet as to whether skin swabbing without anaesthetic is more refined than our improved approach to fin clipping under anaesthetic. The most refined approach will be used.

For genetically modified strains of scientific interest where animals may develop a harmful phenotype, we will first do a small scale pilot study to ascertain the onset and penetrance of the phenotype. This will determine whether increased monitoring or different experimental endpoints are required.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the PREPARE guidelines and all publications will be in accordance to the ARRIVE 2.0 guidelines. We will also keep abreast of the literature to inform our protocols/procedures for genetic alterations, imaging and anesthesia/analgesia and thus ensure that they are performed in the most refined way possible.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We regularly attend zebrafish-specific conferences where any new developments are relayed to the community. We will keep abreast of new opportunities for refinement and reduction in our experiments by continuously monitoring published research to ensure we always apply the best possible statistical methods, animal husbandry and experimental design. We have also developed a close relationship with the NACWOs and animal technicians to ensure that all contribute to implement any new advances in the 3Rs effectively. We will use the NC3Rs website and sign up to their newsletter to stay updated on relevant news and workshops. We will follow the PREPARE guidelines and all publications will be in accordance with ARRIVE 2.0 guidelines.

27. Maintenance and Breeding of Genetically Altered Animals

Project duration

5 years 0 months

Project purpose

Basic research

Key words

Breeding, Maintenance, Cryopreservation

Animal types	Life stages
Mice	juvenile, adult, embryo, neonate, pregnant
Rats	neonate, juvenile, adult, pregnant, embryo
Zebra Fish (Danio rerio)	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To provide a centralised breeding service programme which includes rederivation and the cryopreservation of sperm and embryo's.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Research projects that require the use of animals regularly utilise genetically altered (GA) animals which are often models of disease and can be unique. This project licence will provide to a centralised service for the provision of such animals, provided by experienced

and specialised technicians to support the research projects that require them and help minimise numbers and reduce wastage.

Genetically altered animals are important in helping to discover what causes disease. For example, they may be models with a genetic change that mimics a disease like arthritis, or enable the study of implanted human tumours for testing new anti cancer medicines. Genetic manipulation allows the function of a single gene to be studied in a particular disease, and their use has provided a wealth of knowledge on the regulation and expression of genes and how this can impact the health of humans and animals alike when normal mechanisms don't function properly.

This centralised service licence for breeding and supply of mice to scientific projects is administratively efficient, with breeding controlled to produce the numbers of animals as needed with any spare made available for use by several different (and appropriate) scientific projects.

Cryopreservation, in vitro fertilisation (IVF) and rederivation are also important tools in the management of GA colonies. A rederivation service allows the managed import of SPF (specific pathogen free) animals into the unit, protecting the health status of the facility and of all animals housed within it, and the ability to archive GA strains is also valuable for safeguarding a colony from events such as mutations or genetic drift.

What outputs do you think you will see at the end of this project?

The provision of an effective service for the breeding and maintenance of GA animal models which supports scientific research programmes in the development of knowledge, medicines and treatments of disease for the benefit of humans and other animals.

Who or what will benefit from these outputs, and how?

Research groups and collaborators will benefit from a centralised service that breeds and maintains GA animals on their behalf. Animal welfare is a priority and the high standard of housing, husbandry and care provided by the facility team will ensure minimum wastage, enable tissue sharing and bio- banking where possible, and will ensure rigorous welfare monitoring as part of the service.

A local cryopreservation service can also help to reduce the number of animals bred, and the ability to transport frozen sperm, embryos (and fish eggs) as opposed to live animals will be another benefit from this project licence.

How will you look to maximise the outputs of this work?

Communication with research groups and the facilitation of a genotyping service will help maximise colony use, streamline breeding and reduce wastage. In consultation with end users production will be reviewed to meet requirements whilst minimising excess. Strategies include breeding pauses and timed matings in place of continuous breeding, and cryopreservation of strains.

We will enable tissue sharing and biobanking where possible, will facilitate rederivation in-house to help keep our colonies free from opportunistic pathogens following import from other UK or international facilities. A cryopreservation service for strain archiving and safeguarding will be implemented.

We will also facilitate the import and export of GA animals or gametes to assist with national and international collaborations, and will encourage knowledge sharing to help with prevention of duplication.

Species and numbers of animals expected to be used

- Mice: 56400
- Rats: 100
- Zebra fish (Danio rerio): 5000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are the most commonly used species for genetic modification and is the most utilised GA species at the institution, with anywhere between 50 and 80 strains bred at any one time. We occasionally house GA rats in the facility and whilst we have the ability to breed them, this has not yet been undertaken. The breeding of genetically altered zebrafish can also be accommodated, and we do encourage research groups to look at this species as an potential alternative to small mammals.

In order to support the researchers, we need to be able to supply them with the different life stages required for the work they are conducting.

Typically, what will be done to an animal used in your project?

Mostly breeding and maintenance with some associated processes such as genotyping. Other procedures are used for assisting with maintenance of our SPF barrier and keeping our animals free from pathogens (i.e. rederivation), and processes associated with cryopreservation for archiving and safeguarding purposes.

What are the expected impacts and/or adverse effects for the animals during your project?

Most animals (>90%) will experience either subthreshold or mild affects. Those that experience moderate effects will do so due to the phenotype of the model, though monitoring, intervention and humane end points will be implemented to minimise any suffering.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority of mice will experience either subthreshold or mild severity at most. For those that do experience effects, this will be due to their genotype (associated with the genetic modification), such as osteoarthritis, immune suppression, ageing, and cancer, but they will not be allowed to exceed moderate severity.

For rats, all of them will experience no consequences of their genotype or mild at the most.

Zebrafish will experience either no effects due to their genotype or mild at most, and this will be (for example) stunted growth and reduced fertility. Husbandry practices will be in place to manage these consequences.

What will happen to animals used in this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In vitro work is an important preliminary step for assessing the biological function of a gene or protein and investigations can be performed using cell lines, however the interaction of genes and the regulation that goes on via multiple systems will be absent in vitro. Therefore our researchers sometimes need to proceed from these initial in vitro experiments to test the effects in vivo in a fully functioning biological system. In order to investigate the interaction between all the individual cells, growth factors, molecules etc involved, this work must be performed in animal models and GA animals in particular can be very useful and powerful models to help with this research.

It is hoped that the results of the in vivo studies that our researchers conduct will also contribute to the refinement and development of in vitro models that will be useful in further experiments as opposed to live animals.

Which non-animal alternatives did you consider for use in this project?

Our researchers are always looking for alternatives and the use of human cell lines, mathematical modelling and invertebrates is active and promoted throughout the University. We assist with engagement of our researchers in workshops and other industry events and sources that look at alternatives to the use of animals in research, and these also encourage and enable collaborations with researchers using non-animal alternatives.

Why were they not suitable?

The researchers using the GA animals bred under this licence require the fully functioning biological systems that currently can only be replicated in a live animal but they are always looking for alternatives to this work. Their work with these animals will already have been approved locally or through project licence application.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise

numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers estimated are based on the project licences and research groups that have used this service over the previous 5 years, and also on future predictions. The numbers are an estimate of what may be used of over the course of the next 5 years taking into consideration that most animals will be used for breeding purposes, the maintenance of colonies and supply to other projects. A smaller number of animals will be used to establish clean colonies following import, and for cryopreservation processes (including vasectomy).

Colony breeding will be tailored to individual user needs with an aim to keep numbers as low as possible using best practice breeding strategies. Cryo-preservation will also be used as a tool to reduce numbers when colonies are not in use.

Currently, there are approximately 70 colonies of GA animals in house.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

This licence is intended to provide a service to our research community, by centralising the management of GA breeding colonies and some associated processes. No scientific experiments will be performed under this project licence.

The number of animals used under this licence are determined by individual researcher needs, however we help address reduction through tailored breeding strategies and utilising cryopreservation to reduce overproduction and minimise wastage.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We adhere to the recommendations in "Assessing the welfare of GA mice (April 2006)" and will follow any other Home Office endorsed guidelines and industry best practice to ensure that the welfare of animals used is at the highest levels and numbers of animals used and bred are optimised to reduce wastage and unnecessary breeding.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are the recognised species for work involving genetically altered animals. A variety of models will be maintained under this service licence for use in authorised research

projects investigating infection, cancer, ageing, and other human and animal conditions and diseases. The models used may be reporter or cre lox lines, immunocompromised lines, or models specific to a disease or condition such as the Trp53 knockout (tumour suppressor knockout) mouse, but these models will be the most appropriate for the intended work and that which will cause the least harm. Most animals under this licence will be used for breeding purposes, timed mating for the production of embryos, schedule 1 killing for tissue collection, or maintained until transfer to another project. These animals and those used for the other standard protocols (such as superovulation or embryo transfer) are not expected to experience pain, suffering, distress or lasting harm above mild or moderate severity.

Rats are used more rarely than mice but are needed for some specific projects. For example, nude rats are used in cancer projects as their immunocompromised status allows them to grow subcutaneously implanted tumours (but no tumours will be produced under this project licence), and the ACE2 rat will be used for infection research.

The Zebra fish on this project licence will be used to produce eggs and embryonic stages of fish for use in preliminary studies as an alternative to more developed and sentient animals. Juvenile and adult fish may be used for the purpose of schedule 1 and tissue collection, or transferred to other approved project licences.

Why can’t you use animals that are less sentient?

The projects we supply to determine the species and life stages required and will already be approved to use these models. The actual procedural and experimental work on the animals produced under this licence will be performed under these other projects.

Most of the work conducted under this licence will be for breeding and/or maintenance purposes therefore must include all life stages. Processes associated with cryopreservation or rederivation (superovulation for example) have to be performed on adult mice.

The Zebra fish on this project licence will be used to produce eggs and embryonic stages of fish for use in preliminary studies as an alternative to more developed and sentient animals. Juvenile and adult fish may be used for the purpose of schedule 1 and tissue collection, or transferred to other approved project licences.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All facility processes (whether husbandry or protocol related) are reviewed to reduce harm and improve the welfare of our animals where possible.

Our team have extensive experience and expertise in the breeding and maintenance of GA animals and this is drawn upon to maximise the welfare of the animals in our care. There is a system of monitoring in place to identify phenotype and welfare issues associated with genetic modification, and any remedial care or husbandry processes are documented and regularly reviewed.

The other procedures used (e.g. injection and associated restraint) are refined in line with best practice, and we always perform these procedures in a manner that will cause the least harm. This includes surgical processes (such as vasectomy) for which the protocols

have been reviewed and refined to minimise adverse effects. Associated preoperative and postoperative care has also been optimised to reduce pain and suffering, and we have a standardised (minimum) 7 day post operative monitoring period in place. We always employ a regime of post operative analgesia for any surgical procedures.

We also utilise non-aversive handling for all our mice (mainly tunnel handling) to which they become accustomed, reducing anxiety in the species. Our handling, weaning, genotyping, housing, husbandry

and enrichment processes for all species have been reviewed and refined over many years to improve the welfare of our animals as much as possible within the environment they are kept.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We continue to follow the Animal Procedures Committee's recommendations (now the Animals in Science Committee) in the “Assessing the welfare of GA mice” report (April 2006), and will follow any Home Office endorsed guidelines that may supersede these.

The NC3Rs and other industry platforms and organisations such as RAT (Research animal Training), LASA (Laboratory Animal Science Association), RSPCA and reports from any Joint Working Groups, will be used to ensure we are performing procedures and managing our colonies using the most up to date knowledge and in the most refined way.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are signed up to the NC3Rs newsletters and have access to the NC3Rs regional team to help provide knowledge on advances in the 3Rs.

The project licence holder is a member of management forums (e.g. Animal Welfare and Management Discussion Group) which provide access to knowledge from the UK animal research community on the management of breeding colonies, the care of GA animal models, general welfare and housing of laboratory species, and surgical techniques.

We are kept up to date with regular publications and advice notes from the Home Office and follow any recommendations that come out of this regulatory body.

The project licence holder is an active member of the AWERB and the AWERB Hub network. Regular attendance at workshops, conferences and CPD training will keep the licence holder up to date on advances in the 3Rs.

All of these resources will be used to keep informed about advances in the 3Rs and implementation of them throughout the lifespan of the project licence.

28. Small animal models for investigating bunyavirus pathogenesis and for evaluating antiviral compounds

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Bunyavirus, Oropouche fever, Infection, Antivirals/therapies, Transmission

Animal types	Life stages
Mice	embryo, neonate, juvenile, pregnant, adult
Hamsters (Syrian) (Mesocricetus auratus)	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

1. To study the disease development in hamsters and mice infected with bunyaviruses.

2. To evaluate the efficacy of known and experimental vaccines, antiviral drugs and antibodies in these models.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Oropouche virus (OROV) is a bunyavirus that is responsible for causing Oropouche fever in humans. Initially discovered in the Caribbean in the 1950s, OROV has spread across South America and now causes disease in Brazil, Peru, Ecuador, Argentina, Bolivia, Panama, Colombia and Venezuela.

Historically, the clinical features of Oropouche fever are mild and self-limiting and generally last 10-14 days. Since 2022, a new OROV isolate has emerged in Brazil and has been responsible for a very large epidemic of Oropouche fever. Worryingly, infection with this new isolate has been associated with two fatalities in humans under 30 years of age and up to 10 cases of foetal death. The virus has been demonstrated to replicate more efficiently in human cell cultures and has an apparent increased ability to escape pre-existing immunity to prior OROV infection. It is not currently known why infection with the new isolate is causing these disease manifestations. To date there is no vaccine or antivirus treatment available, and along with gaining an insight into the disease, the new isolate represents a priority area of research.

The virus can infect human or animal cells in culture, and such infection systems are proving useful in advancing our knowledge on the basic processes related to the molecular biology of the virus. On the other hand, the availability of (and accessibility to) animal models is limited. Animal models that mimic human disease are crucial in studies on the processes and factors that lead to the occurrence of the disease, and in the assessment of the potency of antiviral compounds which is an essential step in a drug development pipeline.

Here, our aim is to establish hamster and mouse models to investigate OROV infection and to evaluate in them the efficacy of antiviral compounds or candidate vaccines that are under development both at our establishment and elsewhere.

Once the hamster and mouse models for OROV are established, we can plan comparative studies with historical and the newly emerged isolate to further understand the pathogenesis of Oropouche fever.

What outputs do you think you will see at the end of this project?

Objectives 1 and 2: We hope to gain new insights into the biological mechanisms of bunyavirus infection and spread in the host organism. It is not clear how the virus spreads in the host and what cell types are affected by the infection. We also hope to gain enhanced understanding of the basic pathology in tissues, of how viral and host factors modulate host immune and inflammatory responses, and how the complex interplay between them contribute to Oropouche fever. Comparative studies involving different OROV isolates will provide further insights into the pathogenesis mechanisms from both the virus and host perspective.

Objective 3: There is an urgent need for an effective therapy for Oropouche fever given the ongoing outbreak in Brazil and other countries associated with the emergence of a new OROV isolate. Animal models are essential for pre-clinical assessment of promising new therapeutics. The studies planned herein will allow us to identify drug targets that can be taken forward to the clinic to treat infected patients. Our studies will identify potential drug- and antibody-resistant viruses and assess them in our efficacy model. This will inform future treatment options (e.g. use of drug or antibody combinations) that would mitigate the effect of resistance-associated mutations.

The results of our study will be disseminated in the form of publications, presentations at meetings, and through press releases and social media platforms.

Who or what will benefit from these outputs, and how?

These research outputs will be published in leading interdisciplinary journals so that they can be used by a variety of research and health professionals. The short-term benefits will primarily be specific to scientific researchers investigating the outbreak of Oropouche fever and other bunyavirus-associated diseases. Medium- to longer-term beneficiaries will include drug developers and clinical teams who will be able to use these new insights to further develop novel and more rational therapeutic and patient management strategies to reduce disease burden in South America.

The insights gained will eventually be translated to the human context in collaboration with our partner clinical colleagues in the affected countries. In addition, through interactions with colleagues in pharmaceutical companies, we will actively seek opportunities to use the knowledge gained to further develop novel therapeutic approaches.

How will you look to maximise the outputs of this work?

We will have extensive collaborations with medicinal chemists, virologists, clinical and pharmaceutical partners. We will disseminate new knowledge in the form of publications, presentations at meetings, and through press releases and other social media platforms.

Species and numbers of animals expected to be used

- Mice: 2500
- Hamsters (Syrian) (Mesocricetus auratus): 2500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Small animals such as mouse and hamster have been reported to be suitable for studies with OROV. Standard golden Syrian hamsters are susceptible to infection with the virus and present clinical features resembling those found in human patients. While standard laboratory mice are resistant to infection by OROV, genetically altered mice have been demonstrated to be useful models of bunyavirus infection.

The newly emerged OROV isolate has shown a potential increased ability to transmit between infected humans, an ability to escape pre-existing immunity from prior OROV infection and a potential increase in human cases where the virus is passed from mother to developing baby.

The choice of Embryo, Neonate, Juvenile, Adult and Pregnant life stages of the animals are based on experimental need to allow investigations and characterisation of how the new OROV isolate has gained the new characteristics described.

Typically, what will be done to an animal used in your project?

Typically, animals will be infected with OROV via an injection under the skin or passively through co- housing or close-proximity housing with infected animals. In some cases, pregnant animals will be infected at defined days post-mating to assess the ability of the virus to pass from mother to offspring.

Animals may have blood samples taken to screen for the presence of virus throughout the infection period.

Some animals will be administered with vaccines, drugs, antibodies prior to, and after, infection with OROV. Some animals may be treated with continuous drug delivery to assess the affect on OROV infection.

Animals will be on procedure typically up to 10 days (in some cases this period could be extended up to a maximum of 30 days). In reinfection studies animals will be on procedure for up to 10 weeks.

At the end of all procedures the animals will be humanely killed and tissue and organs will be harvested for analysis as described below.

What are the expected impacts and/or adverse effects for the animals during your project?

Most procedures to be carried out are associated with moderate severity rating.

When animals are infected with viruses, we expect the animal to experience weight loss, ruffled fur and to display signs of infection. We do not expect any adverse signs in any animal to exceed moderate rating. Any animals showing 3 moderate signs, or any single severe sign will be humanely killed. Animals undergoing all procedures will be monitored carefully and regularly by the personal licence holder to minimise distress and suffering.

All substances will be administered at doses known to be non-toxic, based on the availability (in the literature or otherwise) of their toxicity profile. Pilot studies will be used where previous data is unavailable.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most procedures to be carried out are associated with moderate severity rating. For both hamsters and mice, we estimate overall approximately 50% of animals experiencing Mild severity and 50% Moderate. We do not expect any animal to exceed moderate rating. Any animals showing 3 moderate signs will be humanely killed.

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

For the disease pathogenesis and transmission work, only living animals can exhibit the complex interactions between cells in tissues, the immune system and the virus, which together, help determine how the virus can spread in different tissues and organs, and how it causes the disease.

Therefore, animal models that mimic human disease are crucial in studies designed to understand the disease process and how viruses are transmitted from infected animals to either their offspring or other animals. The viruses used in these studies cause illness in humans, these studies will not provide improvements to our understanding of animal disease.

Which non-animal alternatives did you consider for use in this project?

We use cell culture systems to perform the biological analyses of the virus in the context of infection. We also use such systems to perform studies to identify and evaluate potential compounds that can be developed for therapy.

Why were they not suitable?

From an ability to cause disease and spread of viruses perspective, cell-based systems are useful in advancing our knowledge on the basic processes related to the molecular biology and replication of bunyaviruses. They do not mimic many aspects of the complex multicellular environment of tissues and organs of living animals. Only living animals exhibit this.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We propose to restrict the number of animals to a minimum required to provide statistically significant analysis. From previous publications and upon expert advice from bio-statisticians, we will use 3 to 6 animals (i.e. 3 hamsters or 6 mice) per cage (i.e. one experimental unit).

Each condition (e.g. dose of drug/antibody “x”/virus isolate) will be tested in four experimental units, referred collectively as a “group”.

Hence a group is formed by 4 experimental units (12 hamsters or 24 mice placed in 4 different cages).

Each group will be split in two distinct experiments. The condition is therefore tested initially on 2 experimental units (2 cages, 6 or 12 animals) and then repeated independently once in another 2 experimental units giving a total of 4.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We sought advice from biostatisticians to determine the animal group sizing.

Data from literature, previous cell culture and animal work also has informed experimental design. For example, the amount of virus administered or the pre-treatment of animals with anti-interferon (IFN) antibodies to permit infection with some viruses.

These prior data mean a reduction in the number of animals used for infection experiments can be achieved to resolve our scientific questions. This can be further refined as new data are obtained.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will share tissues where appropriate with our collaborators to minimise the number of animals in the study. We will also employ techniques such as blood sampling to allow us to measure changes in virus titre over time. As these steps can be performed repeatedly within individual animals it will lead to an overall reduction of animals required for each study.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use hamster and mouse models in our studies, as they have been reported to be suitable for studies with OROV and other bunyaviruses. Typically, animals will be administered with drugs, antibodies or vaccine candidates prior to, and after, infection with OROV under the animals skin.

Animals will be on procedure typically up to 10 days (in some cases this period could be extended up to a maximum of 30 days). In reinfection studies animals will be on procedure for up to 10 weeks.

At the end of all procedures the animals will be humanely killed and tissue and organs will be harvested for analysis as described below.

The proposed protocols are designed to cause least pain and suffering.

Why can’t you use animals that are less sentient?

The animals chosen are the least sentient animals that are suitable for our studies. Rodents are the least sentient animal that fully recapitulate the clinical signs of disease experienced by mammals (humans).

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All animals will be monitored daily and will be humanely killed if necessary, following veterinary advice. We will use local AWERB and other guidelines for dosing and for blood sampling, establishing clear humane endpoints, monitoring animals frequently, not allowing infections to develop into severe illness.

All laboratory animals will be handled using non-aversive methods as this has been shown to have a positive effect on scientific data due to a reduction in baseline stress and anxiety of the animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow Home Office and NC3R guidelines and those published such as:

Carbone and Austin (2016) Pain and Laboratory Animals: Publication Practices for Better Data Reproducibility and Better Animal Welfare. PLoS ONE 11(5): e0155001. doi:10.1371/journal. pone.0155001.

Percie du Sert et al. (2020) The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. PLoS Biol 18(7): e3000410. https://doi.org/10.1371/journal. pbio.3000410.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Keeping abreast with any new information and guidelines through the NC3R website and through our animal facility. We will continually liaise with the animal facility staff and attend relevant presentations from them to ensure that we are up to date with the information and that they are implemented.

29. Characterisation of normal and malignant haematopoiesis

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

haematology, stem cells, cancer, bone marrow transplantation

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand how blood is produced and how diseases like leukaemia develop from blood. With this knowledge we can work towards making limitless supplies of blood outside the body (for transfusions and transplantations) and identify molecules to target for treating blood cancers.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Our research will primarily focus on two areas: 1) How blood cell production is regulated in non- diseased situations and 2) Understanding disease development. Through this work we will gain an improved understanding of the way in which the normal production and function of blood cells is perturbed during disease development and progression, in order to create new drugs to treat diseases arising in blood cells (such as leukaemia). We also

wish to use our improved understanding of how blood cells expand, in order to make vast quantities of blood cells outside the body to provide virtually limitless numbers needed for use in cellular and gene therapies in particular.

What outputs do you think you will see at the end of this project?

We aim to improve our fundamental understanding of normal blood cell production, in addition to tracking the changes that result during disease. This work will increase our knowledge of how pathways regulating normal blood cell production may be hijacked in disease, and whether these pathways can be modulated either positively or negatively to treat disease.

We will use the new information we gain about the regulators of blood cell production to develop more effective ways to culture blood stem cells (the cells from which all other blood cell types develop) successfully in vitro. These improved methods will be shared with researchers and industry partners all over the world.

We are also working to produce more effective tools to precisely map and manipulate key blood cell types that are involved in normal and stressed blood cell production, and also to create more accurate mouse models of complex disease in blood cancers. Once developed, these resources will be widely shared with other UK researchers and international academic and industrial partnerships. These models will also facilitate a better understanding of a wide range of diseases by allowing cells of the immune system to be identified and targeted (e.g., cancer, infectious diseases, diabetes, etc), benefitting the wider research community.

Who or what will benefit from these outputs, and how?

In the short term, the knowledge and tools gained from these studies will be of interest to the scientific community. Longer term, we hope that clinicians and patients will benefit from our research, through novel therapeutic strategies to treat blood cancers and improved stem cell expansion methods for cell and gene therapies.

How will you look to maximise the outputs of this work?

Results arising from these studies will be published in peer review journals and presented at national and international meetings to disseminate knowledge to scientists and clinicians. We will also promote the sharing of best practice and new methods for reducing animal usage in the blood stem cell field.

We also regularly engage with the local Haematology Support Group, a local group of patients with blood cancers.

We will also partner with our collaborating institutions to centralise resources and expertise, making them available to the wider scientific community and engage with academic colleagues and industry to translate our findings.

Species and numbers of animals expected to be used

- Mice: 3,750

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

In order to study blood cell production and blood cancer development, we need to recreate the complexity of these processes in a whole model organism. We use mouse models as they are warm- blooded mammals that share many features of human physiology, metabolism, immune responses and blood system that are not found in other cold-blooded species such as flies and worms.

Cancer in humans arises from alterations in genes, so to model cancer development we recapitulate the same genetic driver combinations in mice to allow us to study how the cancer progresses and to test novel therapeutic strategies.

For blood stem cell studies of haematopoiesis, the field-standard requires us to demonstrate that a cell possesses the ability to sustain lifelong blood cell production and this can only currently be assayed in a whole organism. We use transplantation models where we transplant blood stem cells into the mouse to understand how (and for how long) the cells and the host environment affect blood cell production.

For these reasons, the mouse is the most appropriate and widely used animal model for studying blood cells and cancer, therefore techniques are well established and findings can easily be integrated with other groups' data.

Typically, what will be done to an animal used in your project?

Typically, animals will experience brief, slight discomfort and no lasting harm from administration of substances by injection using standard routes (typically intravenous, subcutaneous, intraperitoneal). Animals will experience very brief discomfort from blood sampling associated with insertion of a small needle through the skin. They may also be imaged non-invasively, involving anaesthesia used for immobilisation only. Mice are expected to make a good recovery following anaesthesia and no adverse effects are expected.

Occasionally, bone marrow samples may be taken from the femurs, typically twice within a long-term experiment, and only once from each side of the body. The procedure will be performed aseptically, pain relief will be provided, and the mice are expected to recover quickly with no lasting adverse effects. Post-procedure, animals will receive a soft gel diet to encourage recovery and will be closely monitored for any signs of discomfort or infection.

Mice may undergo bone marrow transplantation, which involves injection of donor cells into the tail vein, followed by serial blood monitoring to quantify the number and type of mature blood cells produced. The injection of cells is not directly associated with any adverse effects. Some recipients may first undergo whole body irradiation, but for the vast majority of our experiments, we will use sublethal doses at which we do not anticipate any lasting adverse effects.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice that undergo whole body irradiation before bone marrow transplantation may experience some transient discomfort, such as weight loss for a 2-4 week period, and the mice may be supported with a soft gel diet. Mice receiving high doses of irradiation will either receive a very high dose of bone marrow cells or will receive a small dose of “test” donor cells accompanied by genetically distinct “helper” cells to aid their recovery and may be treated with antibiotics to reduce the risk of infection.

Beyond this initial period, the majority of animals are not expected to show additional adverse effects that impact their general well-being.

Mice receiving genetically modified cells through transplantation may develop blood cancers, which can be associated with fatigue and other clinical symptoms due to anaemia. Animals will be routinely health checked, blood samples monitored for abnormal parameters, and any animal exhibiting clinical signs of cancer will be humanely culled at pre-determined endpoints to minimise any discomfort.

Administration of substances may result in a significant reduction of the immune system. Mice will be monitored closely by blood cell sampling and for clinical signs (e.g. loss of appetite, breathing difficulties, poor movement) during treatment and for 2 weeks following the final treatment timepoint, and may also be given antibiotics to reduce the risk of infection. Any animal exhibiting overt clinical signs, or signs of distress will be humanely culled.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice: 70% mild, 30% moderate.

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to use animal models to understand how the normal balance of blood cell production and destruction is subverted to drive disease and how blood cells interact with their neighbours to promote or suppress diseased cells. The processes of both normal blood cell production and the development of blood cancers are extremely complex and involve many different cell types interacting and communicating with each other, as well as with the microenvironment. These interactions are currently too complex to be replicated in vitro.

It is also not currently possible to study blood stem cells in vitro because the assays thus far developed cannot discriminate stem cells from other blood cell types. Currently, the only assay available for the identification of blood stem cells is repopulation of the blood system after transplantation into irradiated mice. That said, in our last PPL, we made a substantial advance by identifying specific markers on the cell surface in vitro that strongly predict which cells have retained stem cell function. While we still require transplantation to prove that a stem cell was present, we can confidently avoid a large number of experiments not testing cells that do not have these properties.

Which non-animal alternatives did you consider for use in this project?

We use gene expression datasets that we have generated or are already in the public domain to inform our animal experiments. Candidate genes for functional assays are typically assessed extensively in silico and in vitro prior to their testing in animals. Although the only assay currently available for identifying blood stem cells requires transplantation into mice, initial data on new candidate genes and molecules involved in stem cell regulation will be obtained from in vitro culture experiments. We also use human cell lines (including patient derived cell lines) of particular mutations associated with blood disorders to study the biochemistry of the mutations. Also, due to our recent collaborative work in human HSC expansion, we are able to move some experiments into human and avoid mice altogether.

Using this system, we have nearly fully replaced our need for studying human blood stem cell properties in animals by optimising cell culture conditions for the expansion of normal and patient derived blood stem cells and the cells they produce. In future we may still need to validate some findings in mouse models, but for now, xenografts (the transplantation of human blood cells into mice) do not feature in our PPL due to the success of the in vitro methods.

Why were they not suitable?

Recent advances in mouse blood stem cell expansion protocols mean that functional blood stem cells now show improved expansion capacity, but the stem cells still represent the vast minority of cells, and substantial variability exists between single cell expansion cultures. Further optimisation is required to enhance blood stem cell expansion capacity and permit analyses of purified expanded stem cells and we are working to develop robust in vitro assays to expand and detect functional blood stem cells.

Until these in vitro methods are improved however, the only way to precisely identify blood stem cells is by using in vivo stem cell transplantation methods in mice. Therefore, we will continue to use in silico and in vitro methods, as described above, to gain useful insights that support and inform our in vivo studies, but currently these alternatives are unable to recapitulate the complexity of the blood system of the whole animal. Also, we are unable to study the function of various immune cells in the human system in the same powerful way that various reporter mice allow us to do in humans since we do not have an in vivo alternative to study sophisticated genetic control mechanisms (e.g., deletion of specific genes).

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Experiments planned under this licence are similar to those being performed in my laboratory currently, so animal numbers are estimated based on our previous experience. Typical variations from our earlier experiments were used to calculate the minimum number of animals needed to achieve statistically significant results in our future projects.

We maximise data output from the animals we use by performing longitudinal studies and collecting as many tissues as possible at experiment endpoints, making them available to other researchers, or storing them for use at a later time.

As well as this, we are undertaking a number of measures to reduce our animal usage, described below, and therefore our estimated numbers of animals for protocol 1 and 2 of the previous project licence have now been reduced by 25%.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We are implementing several practises that are allowing us to reduce the number of animals being used in this project:

- Power calculations using the 'Experimental Design Assistant - EDA' on the NC3Rs website have enabled us to minimise the number of animals we use for our experiments, whilst still gaining robust and meaningful results.

- We have identified two markers that we can use to enrich for blood stem cells in vitro, providing us with larger numbers of stem cells and reducing the number of mice needed for our experiments. I have also been awarded funding from NC3Rs to continue our work to optimise methods to culture blood stem cells in vitro, to reduce and ultimately replace the need to study these cells in mice.

- We have also developed low cell number proteomics approaches in our lab to reduce the number of animals needed to obtain proteomics data.

- We have established human blood stem cell expansion conditions in collaboration with colleagues which has moved some experiments directly into human cells, instead of using mice.

- The cumulative effect of the above has enabled us to reduce our proposed animal numbers in this project licence renewal by 25% in protocols 1 and 2 of my previous licence.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We use pilot studies with a small number of mice in the first instance, to optimise our experiments and inform on numbers that will be needed for meaningful results to avoid overpowering the analysis.

We source mice from central colonies to avoid redundant breeding and interact with colleagues to share tissues and cells from mice, both within our lab and across labs, with commonly used strains.

We often harvest multiple tissues post-mortem. If we don't need to analyse the tissues immediately, we will freeze them to analyse later or make them available to other researchers working on similar questions.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse is the most appropriate and most widely used model for the blood cell production system and blood cancer development. The techniques are very well established and findings can easily be integrated with our group's data. We are continuously reviewing our techniques and monitoring new developments that lead to a refinement of regulated procedures.

The mouse is also the species in which the technologies to alter genes are best established. We will use mice that contain non-harmful genetic alterations, such as fluorescent reporters, to identify and track different blood cell types in the animal. To induce gene expression or to deplete specific cells, some animals will be given substances by mouth, injection, or through food. Oral gavage or injection may be necessary to induce a rapid change in gene expression, which allows us to study processes that happen within short time periods.

We also use mice with the same genetic changes that are known to cause human blood cancers to accurately model the human disease. All animals are monitored regularly, and the studies ended as soon as we observe adverse clinical signs, to prevent unnecessary suffering.

Bone marrow transplantation is the most refined method for identifying blood stem cells and studying how they produce more mature blood cell types. Irradiation before injection of donor cells can cause transient discomfort for the animals but it is often necessary for the engraftment of the donor cells.

Where possible, we will use sub-lethal doses of irradiation to minimise discomfort.

Why can't you use animals that are less sentient?

Mice are the least sentient species that we can use to accurately recapitulate the complex processes of blood cell production system and blood cancer development. Non-mammalian animals are limited in their use because their blood cell production is too different from that of the human system to provide relevant results. We cannot use embryos or young animals for our studies as the blood system is not mature at these stages, so only adult mice are relevant to study adult blood cell production processes. Most blood cancers also occur in adults and often these diseases require extended periods of time to manifest.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We are continuously reviewing our techniques and monitoring new developments that lead to a refinement of regulated procedures, following guidance locally and nationally, e.g. from NC3Rs. For example:

- When exposing mice to a lethal irradiation dose for bone marrow transplantation, irradiation will be administered as a split dose to reduce the severity of the procedure.

- We will also use c-Kit mutant W41 mice as reconstitution recipients where possible as these require only a single sub-lethal irradiation dose and do not require carrier cells from an additional mouse.

- For our I.V. transplantation and peripheral blood collection protocols we follow LASA guidelines, including the use of the smallest needles possible and applying finger pressure on the soft tissue to stop the blood flow after collection/injection.

- It is our standard practice that needles will only be used once to avoid dulling of the needle, which would cause the animal pain.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

When planning and carrying out our experiments, we follow the published PREPARE and LASA guidelines.

The online Experimental Design Assistant (EDA) from NC3Rs will be used to design experiments where appropriate.

We will also adhere with the ARRIVE guidelines 2.0 when planning, conducting and reporting our animal experiments in publications.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We continually review our processes and will be guided by the NACWO, NVS and Home Office Inspectors. We regularly check information on the NC3Rs website and receive their newsletter, with anything relevant being discussed at our lab meetings. We also attend all forums held by our animal facilities management team, which are always extremely helpful for acquiring information related to the 3Rs and where we regularly benefit from advice from external speakers and learn of the latest advances in best practices.

30. Natural killer cell therapy for cancer

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer therapy, Immunity, Natural killer cells, Immunotherapy, Vaccine

Animal types	Life stages
Mice	adult, neonate, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing

What's the aim of this project?

The aim is to develop new ways of activating natural killer cells to kill cancer.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cancer is one of the biggest causes of morbidity and mortality. There are over 375,000 cases of cancer each year in the UK alone, with over 160,000 deaths (Source:https://www.cancerresearchuk.org/health-professional/cancer-statistics-for-the-uk). Many cancers are thus difficult to treat. Natural killer cells are cells of the immune system that are known to recognise and directly kill cancer cells. They can also help other immune cells to fight cancer.

However, currently they are emerging as a therapeutic entity and it is not well understood how they can best be used to treat cancer. We are aiming to develop new types of natural killer cell therapy and wish to perform the research studies underpinning this objective.

What outputs do you think you will see at the end of this project?

Cancer is one of the major killers worldwide. Immunotherapy is an area of growing interest that offers an alternative treatment to conventional cancer chemotherapy. Currently immunotherapy is focussed on activating T lymphocytes, however there is a growing body of work suggesting that natural killer (NK) cells may be an alternative target for immunotherapy as they work in different ways from T lymphocytes and clinical studies have shown that they have fewer side-effects. The overarching aim of the project is to develop a new type of cancer therapy based upon natural killer cells, which is based upon our fundamental research. The project will develop and test the parameters required for a successful natural killer cell targeting vaccine. To do this we need to take a stepwise approach by developing and testing our therapeutic strategy. During this programme of work we will:

1. develop new tumour models for understanding natural killer cell therapy
2. develop new methods for activating natural killer cells
3. investigate different ways to activate natural killer for immunotherapeutic benefit
4. test the effectiveness of our strategies for targeting cancer
5. identify which cancers are susceptible to our therapeutic strategy
6. identify currently available drugs which may be used in combination with our NK cell activating strategy
7. test how well our cancer therapy works compared to other cancer therapies

We will thus gain new knowledge in natural killer cell activation and the protocols by which they can be stimulated. We will also identify how this activation relates to an anti-cancer immune response and a method by which it can be translated to the clinic for patient benefit.

Who or what will benefit from these outputs, and how?

In the short-term the scientific community will benefit from our research. In particular the immunology and cancer immunology specialities will gain new knowledge relating to how natural killer cells can be activated and how this can be used for immunotherapy. Our strategy is unique at present as it is based upon a scientific discovery that my research team have made and on our on-going work showing that natural killer cells can be activated using a vaccine-based approach. We have been refining this technology to help make the natural killer cells as active as possible against cancer. We will present these findings at academic meetings and also publish them in the scientific press. We are currently working on manuscripts that we hope to publish in the next two years. Understanding the best methods for using natural killer cells to treat cancer in the clinic is our main objective and based on our current results we are hoping to test our first vaccine in the clinic in the next 3-5 years. We are also developing further vaccines that target natural killer cells to treat cancer and this will be done over the next five years.

How will you look to maximise the outputs of this work?

The works will be presented to the academic community at national and international meetings and also through the scientific literature, targeting open-access journals, to disseminate the findings. We will also disseminate the work locally through academic meetings and presentations. Significant findings can be disseminated through the University press office and also public engagement activities such as "Pint of Science ". Negative findings will be published on BioRXiv.

Species and numbers of animals expected to be used

- Mice: 2000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using mice as these are the least sentient animal that we can perform these experiments on. The mouse has a well studied immune system to allow parallels to be drawn with humans. Human and murine NK cells share key functions such as cytotoxicity, cytokine secretion and anti-tumour responses, and mice have been previously used to develop NK cell therapeutics prior to clinical use. As a well studied animal model, sufficient reagents are available to analyse responses within the murine immune system to the level required to make these experiments insightful. Furthermore mice can be genetically modified to express the human genes that we are targeting with our vaccination strategy. Human cells can also be infused into some strains of mice and these can be used to test our vaccination strategy. This provides a more faithful model in which to test our vaccine strategy. In general we are using adult mice as these are the most resilient life stage and allow the experiments to be standardised. Furthermore we are studying human adult cancer. In general mice will be studied between weeks 12-16, well before age-related effects will be observed. However in order for mice to fully accept a human immune system they may need to be reconstituted as the neonatal stage and this forms a small part of our project.

Typically, what will be done to an animal used in your project?

In a typical experiment mice will be injected subcutaneously with a tumour cell line and then will have natural killer cells infused intravenously via the tail vein, neither of which require anaesthesia. They will then receive two or four DNA or RNA vaccine injections into the thigh muscle. Each injection will be three to four days apart and alternating thighs used each time. Tumour growth will be monitored by measuring using callipers on alternate days and mice will be killed humanely when the tumour reaches a specific size or if another a humane end point is reached. The duration of the experiment will depend on the tumour growth parameters but overall we expect these experiments to last from 3 to 6 weeks.

At the end of the experiment the mice will be analysed for activation of immune cells in their different organs.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals may experience local irritation to injections. This is usually mild and passes within 24 hours. Some of the injections, such as the the Toll-like receptor agonists, may have more generalised side effects of longer duration such as shivering, erection of the fur, reduced motor activity, hunched position, lack of responsiveness and shallow breathing. Animals exhibiting these signs will be warmed and monitored closely. These effects usually pass within 1-2 hours. This may affect up to 10% of animals. Animals treated with the chemotherapy agents that we plan to use may experience weight loss and diarrhoea. If symptoms are prolonged the animals will be humanely killed to alleviate suffering.Some tumour models induce ulceration, which can be a source of discomfort and distress. Animals experiencing ulceration will be carefully monitored and killed if any signs of distress are noted. Rarely mice may experience graft versus host disease. This is unlikely if given natural killer cells alone but more likley in a humanised mouse model. In general we will aim to use a natural killer cell infusion, rather than a humanised mouse model where possible, to limit harms. Impacts will be minimised by careful monitoring, taking advice from the NVS and killing before significant harms arise.

We anticipate that by operating within the guidelines for blood drawing and tumour challenge and also within previously studied dosing levels of any drugs given to the animals, then with careful monitoring the animals are not expected to experience significant adverse effects related to these procedures.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We anticipate that 90% of animals will have severity scores of mild, and that up to 10% will have a severity score of moderate, based on our previous experience.

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to use animals to develop and test our vaccine strategy. The ultimate goal of this project licence is to devise a new way to treat human cancer, but this needs development and refinement in animals before we can move to human studies.

Which non-animal alternatives did you consider for use in this project?

We are gaining as much as information as possible using in vitro cell culture experiments using cell lines and human blood cells, prior to evaluating the use of the vaccine in

humans. Our vaccine is a novel first in class therapeutic that targets natural killer cells. We have performed a literature search to identify alternatives to this, such as in silico modelling or organoid culture, and have not found one which can test the effect of a vaccine on natural killer cells.

Why were they not suitable?

We have not identified a system that can faithfully recapitulate the response to a vaccine that targets natural killer cells, and so have been unable to identify a suitable in silico or in vitro model for this work. In general, these types of model systems do not recapitulate a whole animal experiment that is required prior to using a potential therapeutic in humans. This is because generating an immune response requires multiple steps and the interplay of many different immune cells in a co-ordinated fashion within a localised environment. This interplay cannot be readily or faithfully reproduced in tissue culture conditions to the standards required to inform a clinical trial. Furthermore, unanticipated toxicities cannot be readily identified in in vitro model systems.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the numbers based on our previous usage of around 300 animals per year. This is because whilst we are reducing the numbers of animals used to test an individual vaccine therapeutic, we are testing some different anti-cancer vaccines that we are developing. Concurrently we are developing ex vivo methods to test our therapeutic strategy with a view to reducing animal numbers.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In the design phase we have used inbred mice to reduce intra-group variability and allow reduced mouse numbers for experiments. We have designed experiments using the fewest animals consistent with obtaining statistically valid results as determined from power calculations made using the online NC3Rs Experimental Design Assistant and a two group comparison with complete randomisation. We will also store material from our experiments so that they can be used to interrogate our research questions in more detail and inform experiments prior to using further animals. We have also reduced the number of animals required by using an immunodeficient mouse model which accepts human tumours and human natural killer cells and vaccinating those animals. Previously we had to vaccinate one strain of mice and transfer the natural killer cells from those mice into the immunodeficient mouse. Our new strategy effectively reduces the number of mice that we need to use to test the response to human tumours by half.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will carry out small pilot experiments to assess simple factors such as dose or route of administration prior to performing larger experiments. Where multiple inter-relating parameters are to be evaluated, we will use a factorial design for experiments in order to prevent use of excess mice as controls. Furthermore we will make optimal use of multi parameter analysis e.g. flow cytometry or RNAseq so that as many different parameters as possible can be analysed within a single sample.

Additionally we have developed more faithful ex vivo spheroid models of cancer that we can use to test the immune response to a vaccine and its anti-cancer effect. This allows us to reduce the numbers of mice that are given tumours. We will also make full use of stored samples from our vaccination experiments to answer our research questions prior to performing further experiments in live animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use genetically altered and immunodeficient mice as our animal models. These are necessary as our vaccine targets uniquely human proteins with uniquely human ligands. The genetically altered mice contain the ligands for our vaccine strategy and the immunodeficient mice accept human tumours.

We will test the following methods:

1. injections of DNA/RNA/cytokines/antibodies/toll-like receptor agonists delivered by subcutaneous, intramuscular, intraperitoneal and intravenous routes

2. injections of tumours by the subcutaneous or intravenous route

3. injections of immune cells delivered by the intravenous route

4. immunodeficient mice reconstituted with a human immune system

We will select the most appropriate route to deliver the therapy that causes the animal the least distress. In our previous experiments we have not observed lasting harm to the animals using these methods.

Why can’t you use animals that are less sentient?

Mice are the least sentient mammal species with an immune system similar to humans that we can use. This similarity means that mice represent a relevant animal model for these studies and the clinical successes now being reported using immunomodulatory drugs against cancer were dependent on data arising from such murine studies. Murine

cancers are well characterised and the widespread availability of commercial reagents allows direct comparisons between mouse and human immune systems.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Environmental enrichment, good husbandry and frequent monitoring ensure high welfare standards. Few adverse effects are anticipated but, should any occur, rapid steps will be taken to ameliorate them or humanely kill affected animals. All animals will be maintained by qualified and experienced animal technicians who are familiar with the models.Mice will be handled using non-aversive methods e.g. not picking up by the tail, but moved using a tunnel or a cupped hand.

Any animals which are anticipated to be nearing a defined end-point, or for which a defined end-point is not yet established, will be monitored more closely. Should a technician find an animal that has reached an end-point the animal is either immediately humanely killed or the PIL holder is informed that the animal is required to be humanely killed immediately.

We have established end-points for humane killing before pain/distress occurs, based on accepted guidelines. Our tumour models are predominantly subcutaneous in nature, allowing easy monitoring of tumour size. Experiments will therefore be terminated before tumour growth limits behaviours or causes distress. Occasionally, following therapy a subcutaneous tumour resolves from the inside out giving the appearance of ulceration; we have adopted a scoring system to ensure that these are managed with minimum adverse effects to the mice. For intravenous tumours we will use a timepoint before animals become distressed by careful titration of cell number and pilot experiments. While the maximum severity limit for much of the work to be conducted under this PPL is set as ‘moderate’, through experience and good management of the mice, we have found that under our existing PPL that the actual severity of more than 90% of experiments is ‘mild’.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the NC3Rs as a resource for our animal studies and experimental design. We will use information available in the NC3Rs website (https://www.nc3rs.org.uk/3rs-resources). Guidelines contained there include “Responsibility in the use of animals in bioscience research” and the “ARRIVE” guidelines for reporting the use of research using animals. We will also use information from Norecopa and will follow PREPARE guidelines (https://norecopa.no/prepare) for all animal experiments.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed through the regular meetings of the animal facility and the user groups which occur every 3 months. Additionally, we receive e-mails to update us about changes in policy or practice. We will also check on-line databases to identify any changes such as the NC3Rs and Norecopa (including Altweb) web pages. Any changes will be implemented directly through the experimental design, and if necessary through a project license amendment.

31. Assessing Insulin Resistance Prevalence in Sows at Late Gestation: Implications for Sow Production Performance and Piglet Growth

Project duration

2 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes.

Key words

Pigs, Insulin resistance, Gestation, Lactation, Glucose

Animal types	Life stages
Pigs	pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the project's objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To investigate the prevalence of insulin resistance in sow populations and its correlation with pre- weaning piglet performance.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Currently, there is no consensus on the specific thresholds of insulin resistance (IR) in sow populations that detrimentally affect pre-weaning piglet performance.

Moreover, the existing literature does not provide substantial evidence regarding the prevalence of IR levels that are deleterious to the health of the sow and her offspring.

While IR is an important adaptational mechanism to ensure adequate partitioning of glucose for foetal growth and development, there is variation in the degree in which IR is found in reproductive sows. Furthermore, if this pathological IR exists in the sow population, characterisation of the detrimental effects of offspring is required. There is evidence that the degree of insulin resistance in sows is associated with pre-weaning mortality in piglets (Kemp et al. 1996).

Therefore, assessing the prevalence of IR in reproductive sows in late gestation is essential for understanding production performance and mitigating the detrimental impacts of IR on both the sow and her litter.

Kemp, B., Soede, N.M., Vesseur, P., Helmond, F., Spoorenberg, J., Frankena, K., 1996. Glucose Tolerance of Pregnant Sows Is Related to Postnatal Pig Mortality. J. Anim. Sci. 74, 879-885.

What outputs do you think you will see at the end of this project?

The outcomes of this research can be used for a targeted approach for the management of reproductive sow nutrition and health. By establishing a correlation between IR in sows and production performance and litter health, we can tailor diets for gestation and lactation to mitigate adverse effects associated with pathological IR. This research serves as the foundation for a more targeted approach to diet formulation, potentially enhancing sow and offspring well-being. This research may contribute to the development of management practices and diets tailored to insulin-resistant (IR) sows, ultimately promoting better reproductive outcomes and overall herd health.

Who or what will benefit from these outputs, and how?

The outputs of this study will benefit various stakeholders involved in sow management and agricultural practices. Commercial pig farmers stand to benefit from the insights gained, as implementing optimised nutritional strategies based on the study's findings could lead to improved overall herd health, productivity, and profitability.

How will you look to maximise the outputs of this work?

The results obtained from this study will be used as a foundational study to design follow on experiments to investigate the mitigation of IR through dietary manipulation/supplementation. However, once the prevalence of IR at a population level is established, this will guide future research and may encourage alternate management and nutritional strategies based on the prevalence rates or individual IR profiles of sows. The results of this research will be published in peer-reviewed journals. The company we are collaborating with have a strong record of publishing their funded research.

Species and numbers of animals expected to be used.

- Pigs: 165

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Reproductive sows have been selected as this ensures the results are applicable to commercially farmed pigs whilst addressing the aim of the experiment. The animals chosen at this life stage represent a crucial segment of the commercial pig farming industry. Reproductive sows are central to breeding and production cycles. This experiment addresses a key stage in pig production as the nursing performance of sows during lactation impacts the performance of her litter. Furthermore, by examining multiple life stages, from gestation through to lactation and the pre-weaning period, the study can capture the full spectrum of effects that gestational IR may have on sow production and performance.

Typically, what will be done to an animal used in your project?

The experiment will be run over multiple batches (~55 per batch) with 165 individually tagged sows (including gilts) (Large white x Landrace). The sows will have a blood sample collected after entry to the farrowing house, 12 hours post last feeding. With the exception of the blood sampling, sows will be subjected to all normal husbandries, management and veterinary procedures deemed necessary by the management of the farm and/or their nominated veterinary consultant.

What are the expected impacts and/or adverse effects for the animals during your project?

Blood sampling: Possible adverse effects include short-term discomfort whilst being restrained as well as at the injection site during and immediately after collection of blood.

However, home office trained technicians will complete the sampling and therefore will ensure the animals comfort and minimise stress. Samples will be collected using aseptic techniques. All pigs will be monitored to ensure infection of the injection site does not occur, by carrying out daily health checks on all dams for the duration of the trial (from day of entry to the farrowing house until weaning).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild severity for all dams (165) within the trial.

What will happen to animals used in this project?

- Kept alive at a licensed establishment for non-regulated purposes or possible reuse
- Rehomed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.
Why do you need to use animals to achieve the aim of your project?

The aims of this experiment are centred around determining the prevalence of IR within reproductive sow populations, with the ultimate objective of improving the health and pre-wean performance. Such research necessitates the use of pigs as the primary subjects.

Furthermore, the insights gained from establishing the prevalence of pathological IR in sows can serve as the foundation for developing tailored dietary strategies in the context of commercial farming practices. Therefore, due to the specificity of the research objectives and the practical implications of pig farming, using pigs as research subjects is both necessary and appropriate.

Which non-animal alternatives did you consider for use in this project?

No non-animal alternatives were considered for use in this project as the mechanism to be studied in this project specifically relates to pig production.

Why were they not suitable?

Achieving our aims will involve investigating complex physiological interactions in the host and also monitoring their production pre-wean performance. Using sows will offer a comprehensive multigenerational model which is a level of complexity that cannot be replicated in isolated cell cultures or artificial settings. Furthermore, the use of sows is essential for accurately determining the true prevalence of IR within a sow population.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies.

Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Previous work on a sow herd with comparable genetic makeup found that 18% of sows had a significant problem with their glucose metabolism (Kemp et al, 1998). Due to the limited literature in this area we have used this figure as a basis and expect between 12% and 24% with insulin resistance in the sow herd. We have calculated that for a prevalence of 18%, the required sample size is 165 for the margin of error or absolute precision of ±6% in estimating the prevalence with 95% confidence.

With a projected prevalence of 18% in 165 dams, we estimate that approximately 30 insulin resistant dams may be identified.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In order to establish a prevalence rate of insulin resistance (IR) and correlate IR with pre-wean performance we need to ensure enough sows are sampled, to validate the prevalence rate and also have enough IR sows to correlate any pre-wean performance differences. We have considered potential loss/attrition of 4%. With this sample size, the anticipated 95% confidence interval is (12%, 24%). This sample size is calculated using a published prevalence rate calculator. With an IR prevalence rate of 18%, we expect to have 30 dams with IR. Based on a power analysis, 26 IR sows would allow us to detect a 6% difference in born alive litter weight and an 8% difference in numbers born alive. A biostatistics professor who has taught at a masters level and is well-trained in experimental design was consulted on these figures. Fewer animals would not provide sufficient rigour to detect the expected differences in born alive or pre-weaning mortality based on the natural variation in these parameters.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Pregnant dams (Large white x Landrace) from the same commercial sow herd will be used. Pre-wean production performance, including number of pigs born, birth condition, birth weights and litter growth for 4 weeks will be recorded for all sows. As well as recording the pre-wean production performance, body weight, parity, condition, P2 and feed intake throughout the study will be recorded. This will allow us to mitigate the effects of confounding variables and explore correlations.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project will take place under standard commercial pig farming conditions, using pregnant sows. To measure insulin resistance, we will use a single blood sample to evaluate for glucose and insulin levels. We will use two kits: one for glucose (QuantiChromTM Glucose Assay Kit or similar) and one for insulin (RayBio® Porcine Insulin ELISA Kit or similar). We will use a method called the Homeostasis Model Assessment (HOMA) to evaluate insulin sensitivity. This involves a simple calculation using the levels of fasting insulin and glucose in the blood. Using this method helps us to gather the necessary data with a single mild procedure per animal. Blood samples will be taken by trained and licensed personnel to ensure pain and suffering is minimised. The use of commercial sows ensures the research is applicable for commercial pig farming. The proposed analysis will fulfil the scientific goals of the project through a single

mammary vein blood sample. By adopting this method we are performing the lowest number of procedures required to deliver the work.

Why can’t you use animals that are less sentient?

Given the need to understand the true prevalence rate within a sow herd in the UK it would not be appropriate to use any other species than commercially reared pigs.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The regulated procedures involved will not cause pain, suffering or lasting harm more than mild severity as these procedures will be carried out by fully trained staff that possess a Home Office Personal Licence. In line with NC3R guidance, if a blood cannot be taken after 3 attempts the animal will go without a sample collection.

During the entire research trial, all pigs will be health checked daily to ensure the health of the pigs is maintained. Any pigs showing signs of ill-health, as determined by trained research technicians, stockman and/or the veterinarian, will be treated with relevant medication or euthanised appropriately. Treated animals will be assessed daily and discussed with the Named Veterinary Surgeon (NVS) when required. A dedicated pig veterinary specialist from the commercial farm will also be available in these situations.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The procedure for blood sampling from the mammary vein in lactating sows, as described by Scollo et al., 2019, involves applying EMLA cream (or similar) to the vein area for local anesthesia, followed by the insertion of a sterile needle to collect the required blood volume (up to 10ml). Pressure is applied to the puncture site post-collection to prevent bleeding. PIL holders at the facility were taught this procedure by the veterinary practitioner for the farm. The mammary vein blood sample method is a refined technique for blood sampling sows in late gestation as it does not require snare restraint.

Compared to jugular vein, mammary vein sampling has significantly fewer vocalisations during collection (2.94% vs. 94.12%; $P<0.001$).

NC3Rs guidance on blood sampling pigs (non-surgical) will also be followed.

Available at: https://www.nc3rs.org.uk/3rs-resources/blood-sampling/blood-sampling-pig#anchor_4.
Scollo, A., Bresciani, C., Romano, G., Tagliaferri, L., Righi, F., Parmigiani, E. and Mazzoni, C., 2019. A novel blood-sampling technique in lactating sows: the mammary vein route. The Veterinary Journal, 254, p.105397. As well as the advice regarding volumes presented by Swindle MM (2010). Blood collection in swine.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The project license holder and personal licence holders related to this licence regularly check the NC3Rs website and read the regular emails received as part of the Tech3R and

newsletter email list from NC3Rs as well as actively look for advances in the area that could effectively advance the research. The NC3R’s visited the pig facility in March 2023.

They had a tour of the facilities which opened up discussion and has allowed us to develop a line of communication for advice.

32. Orthotopic Tumour Models for Therapy of Advanced Cancer and Fibrosis Models

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Metastasis, Tumours, Fibrosis, Novel drugs, Imaging

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to utilize the most clinically relevant animal models to understand the role of specific genetic abnormalities causing cancer development and progression and the role of fibrosis within the local environment of cancer cells in mice. The main objective is to evaluate novel therapeutic approaches for advanced cancers and chronic diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

On average someone in the UK is diagnosed with cancer at least every 90 seconds, which totals to almost 400,000 new diagnoses per year. Cancer incidence in the UK has risen by 40% since 2002, and by 19% only in the last decade. These numbers highlight the urgent need for improved cancer treatments. Cancer metastasis is the major cause of cancer

morbidity and mortality, and accounts for about 90% of cancer deaths (as reported by CR UK, full information can be found at https://www.cancerresearchuk.org/health-professional/cancer-statistics-for-the-uk).

Currently, the NHS is facing a huge challenge in relation to cancer services. For many years, published figures have shown that tens of thousands of people are waiting too long for a diagnosis or vital treatment across the UK. This increases the incidence of advanced cancer cases with established metastases that are difficult to treat. Thus, there is a clear need to develop agents that act specifically on these metastases to increase patient survival.

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive, and terminal interstitial lung disease with a median survival of 3–5 years. Over the last decade, there has been a significant increase in the incidence and prevalence of IPF worldwide.

Databases estimate that there are 6,000 new cases/year and >32,000 patients living with IPF in the UK. With IPF accounting for 1 in 100 deaths in the UK and increasing rates of hospital admissions worldwide, it has become a major health concern.

Currently, there is no cure that would reverse damage to the tissues caused by fibrosis. Our hope is to find a way to prevent and delay the advancement of this disease in patients.

We have discovered novel inhibitors that are active in primary tumours and have anti-metastatic activity. We aim to assess their efficacy in lung and liver fibrosis models as requested in this application, with a view towards clinical use in unmet need of fibrotic conditions such as IPF, cystic fibrosis and liver fibrosis, as well as addressing the fibrotic contribution to primary and metastatic cancer progression.

What outputs do you think you will see at the end of this project?

We are discovering new therapies targeting genetic alterations associated with tumour growth, tissue invasion, cancer spread and also the cancer’s blood supply through new blood vessel growth (angiogenesis) on which sustained growth and the opportunity to disseminate via the blood stream depends. We also aim to develop models to target chronic diseases, such as pulmonary or liver fibrosis. We anticipate that this will generate a knowledge and new technologies to treat metastasis and chronic diseases, that will be directly transferrable to the clinic. The most promising agents discovered and assessed by us will progress to clinical development and clinical trials in patients suffering from cancer and/or chronic diseases.

Who or what will benefit from these outputs, and how?

Once cancer spreads (metastasis), cure rates significantly diminish and over 90% of cancer deaths are due to secondary cancers at sites in the body other than the origin.

We will model both common and rarer cancers which are hard to cure and also their major sites of metastasis to ensure that our new drugs are capable of tackling these unmet clinical needs. While targeted therapies have shown some promise, the development of drug resistance and the need for rationally designed combinations of drugs is becoming a major issue which will be addressed in this licence.

We will assess the efficacy of agents (on primary tumours and/or metastases) in relation to their concentrations in blood and/or tumour to inform the optimum starting dose and schedule in man. The result will usually be obtained within 120 days.

In parallel, we will discover quantitative biomarkers of response which help us to understand determinants of sensitivity or resistance and to confirm that efficacy is tightly linked to the desired mechanism of action.

This knowledge and the technologies we discover in our models is directly transferrable to the clinic. We also check normal tissues at autopsy and aim to define the 'biologically effective dose': the minimum dose of the drug that gives therapeutic benefit without significant adverse effects. The most promising inhibitors will proceed to clinical development and trials in cancer patients.

How will you look to maximise the outputs of this work?

Our aim is to exploit new targets arising from our earlier established research portfolio.

This means a focus on the earlier stages of drug discovery, with activity on higher-risk, innovative targets and approaches that industry is unlikely to undertake. We seek to accelerate the progress of new medicines in indications of cancer and chronic diseases.

We are specifically studying metastasis (the major cause of cancer deaths) and its response to new therapeutic agents.

Our tumour models are widely used for drug discovery projects within our research team.

We have had key collaborations with pharmaceutical companies. These partnerships enable us to develop new drugs and move them into clinical trials as efficiently as possible.

The companies take on the responsibility of regulatory toxicology studies and formulation development. We also benefit from being situated in the state-of-art facility in a large UK bioscience campus, with many local companies offering a variety of technologies that support our progression.

We have published extensively, including many papers, reviews, and book chapters on refined methods both for both animal and cell-based studies and techniques for studying mechanisms of tumour invasion, angiogenesis, metastasis, and their inhibition. Our work has also resulted in several patents, and we will bring more of them in due course.

Species and numbers of animals expected to be used

- Mice: 8500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We utilise the most clinically relevant animal models to understand the role of specific genetic abnormalities causing cancer development and progression and the role of fibrosis within the local environment of cancer cells in mice. Mice share many common genetic features and, to certain degree, many biological processes too, with humans. They experience many of the same diseases as human and have the same types of organs and bodily systems. Additionally, they are cautiously assumed to be the lowest sentient species. For these and many other practical reasons they are appropriate for in vivo drug discovery studies and are widely used for this purpose.

We use adult mice which are around 2 months old to ensure minimal influence of any diseases and malformations and spontaneous tumour growth associated with ageing of this species. Most of our work is carried out using well-characterised human tumour cells, grown in the appropriate anatomical site in naturally immunodeficient mice to avoid tissue rejection. This enables us to study human cancers in the correct tissue microenvironment.

Typically, what will be done to an animal used in your project?

The mice are maintained in ventilated cages using sterile food and bedding. All procedures are carried out in laminar flow cabinets to avoid infections.

The vast majority of mice in the project will carry some form of tumour – injected into specific organs, such as the breast, pancreas, liver or under the skin; whilst some mice will have the cells injected into a vein to allow spread to the lungs, or in organs where metastatic tumour cells spread naturally.

Furthermore, mice will be treated with new drugs (typically orally, or by injection), appropriately to the cancer types under study. Where possible, imaging will be used to track tumour size and location in the body, and comparisons will be made between control and drug-treated mice. Mice will be injected, by one of a number of routes, with novel inhibitors of tumour growth and/or metastasis that have been shown to be effective in the laboratory in vitro in cell cultures and need to be assessed to see how they act in a whole animal. To allow us to make these measurements we will use imaging methods similar to those used clinically, whilst the mice are anaesthetised to monitor effects on tumour growth. These mice may also be injected with harmless chemicals that aid the imaging process and/or further postmortem tissue analysis.

We may also take blood samples from the tail vein for measurement of blood-borne chemicals and concentration of the novel drugs. Some mice may also be killed humanely whilst anaesthetised in order to obtain larger blood volumes and tissue samples for microscopic observation.

A portion of the mice will be used to study the processes of lung and liver fibrosis.

The agents used to induce the fibrosis will be applied by one of the well-developed and described methods under recovery anaesthesia and mice will be closely monitored for any signs of discomfort and/or pain. These can be treated following the guidance of NACWO and NVS.

All the mice will be killed by humane methods at the end point of the experiments.

What are the expected impacts and/or adverse effects for the animals during your project?

We are constantly working on procedures to minimise actual or potential pain, suffering and distress of lasting harm and /or improve animal welfare in situations where the use of mice is unavoidable. Each of our protocols includes provision for the appropriate anaesthetic and analgesia regimes as well as appropriate humane killing methods, following NVS and NACWO's guidance.

In the models exploring cancer metastasis, in the majority of cases, mice would be expected to experience only moderate levels of discomfort as the tumours they carry would not make a significant impact on their general health and wellbeing, and the majority of other procedures (non-invasive imaging, injection of therapeutics), will generally result in no more than transient discomfort and no lasting harm. In cases where the tumour cells will need to be injected into specific organs, the procedure will be done under recovery anaesthesia. Animals are to be inspected daily for loss of body condition and signs of significant suffering, in accordance with the appropriate guidelines. Any animals displaying signs of considerable discomfort, exhibited by showing signs of pain, weight loss and/or abnormal behaviour will be referred to the veterinary specialist and we will follow their advice regarding further care, which may include local anaesthesia, using anti-inflammatory agents and enhanced diet. In the unlikely cases, whereby animals experience acute adverse reactions to the procedures (ex. becoming pallid, showing increased or laboured breathing, inactivity), they will be killed using humane methods.

In the pulmonary fibrosis model, the introduction of an agent inducing fibrosis is believed to cause transient discomfort at the point of administration, due to the pulmonary drug delivery. To reduce this, it will be administered under the recovery anaesthesia. In liver fibrosis however, it is likely that the agent used to induce the fibrosis will cause transient pain due to possibility of mild peritoneal inflammation, which will be managed by local anaesthesia, and local inflammation which will be managed with advice from the NVS.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

In the cancer and metastasis models, approximately 90% of mice would be expected to experience only moderate levels of discomfort, as the tumours they carry would not make a significant impact on their general health and wellbeing, and the majority of other procedures (non-invasive imaging, injection of therapeutics), will generally result in no more than transient discomfort and no lasting harm. Up to 90% of these mice will be treated with new drugs appropriate to the cancer types under study.

Where possible (up to 80% of mice), imaging will be used to track tumour size and location in the body, and comparisons made between control and drug-treated mice.

To allow this, the animals will be briefly anaesthetised to monitor effects on tumour growth in 80% of the mice. Furthermore, 40% of the mice will also be injected with chemicals that aid the imaging process. We will also take blood samples from the tail vein for measurement of blood-borne chemicals and concentration of the novel drugs in 15% of

mice. A further 10% may also be killed humanely whilst anaesthetised in order to obtain larger blood volumes and tissue samples for microscopic observation.

Up to 1,600 of the mice will be used to study the processes of lung and liver fibrosis, which influences the implantation of circulating cancer cells. We expect that 90% of mice will experience only moderate levels of discomfort in the course of the studies.
In the liver fibrosis model, it is likely that the agent used to induce the fibrosis will cause transient pain (managed by local anaesthesia) and local inflammation (managed with advice from the vet).

In all studies, should the animals experience acute adverse reactions to any of the procedures, they will be immediately killed using accepted humane methods.

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Human cancers develop in 3-dimensional (3D) space within specific tissues in the body.

Each tissue provides a unique growth environment which cannot be adequately modelled in 2D cell cultures grown on plastic dishes in the lab. Whilst the 3D in vitro disease models have been considered and tried in our lab, they were deemed not appropriate for evaluating the efficacy of our novel drugs for the primary and metastatic tumours. Cells grown in the lab (even in special 3D setup) are provided with constant, optimal levels of oxygen and nutrients and mostly grows at a similar rate. This is not the case for tumours grown in the body and this variability can significantly influence responses to therapy. In addition, tumorigenesis and tumour growth is not an isolated event and it impacts the whole organism in significant ways that need to be taken in account whilst testing novel, potential anti-cancer agents. Metastasis in particular (the major cause of treatment failure) is exclusively an in vivo phenomenon, as tumour cells from a primary cancer must access the blood circulation to spread around the body and colonise new organ sites.

This is also true in the case of fibrosis events, as it encompasses development of fibrous connective tissue in the 3-D environment of the organs, as a reparative response to injury or damage, specifically when the excess tissue deposition occurs as a pathological process.

Similarly, the effects of drugs must be assessed in vivo to determine that adequate levels are achieved in tumour tissues, that adverse effects on normal tissues are minimised and that efficacy tracks with effects on indicators of tumour growth.

Which non-animal alternatives did you consider for use in this project?

We aim to first carry out extensive studies in vitro to mimic as many of the basic cellular processes as possible before moving in vivo. We have developed a battery of high throughput 2D and 3D in vitro assays of tumour cell proliferation, migration, invasion, and enzyme activity (processes involved in metastasis and in fibrosis) and we also use endothelial cells (which form blood vessels) in multiple surrogate angiogenesis assays. All compounds are first assessed in tissue culture for suitability for assessment in mice using both simple tumour cell monolayers and sometimes more complex 3D functional assays (e.g. invasion). Failure at any of these stages, limits the number of compounds going forward for assessment in animals.

Why were they not suitable?

Due to the very variable 3D nature of human cancer, the homogenous 2D cell culture model cannot provide the optimal environment in which to study responses to therapy.

Metastasis in particular (the major cause of treatment failure) is exclusively an in vivo phenomenon, as tumour cells from a primary cancer must access the blood circulation to spread around the body and colonise new organ sites. The effects of drugs must be assessed in vivo to determine that adequate levels are achieved in tumour tissues, that adverse effects on normal tissues are minimised and that efficacy tracks with effects on indicators of tumour growth.

Similarly, fibrosis encompasses development of fibrous connective tissue in the 3D environment of the organs, and to date there are no appropriate in vivo models to study the efficacy of drugs reducing the progress of the disease.

Mice are the lowest sentient species that are appropriate for in vivo drug development studies and are widely used for this purpose. Most of our work is carried out using well characterised human tumours, grown orthotopically (i.e. transplanting cells/tissues/organs into their normal/native place in the body) as xenografts (i.e. transplants made to individual from another species) in innately immunodeficient adult mice (i.e. mice with defects in one or more immune components) to avoid tissue rejection. This enables us to study human cancers in the correct tissue microenvironment (albeit in a mouse host).

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

On a typical drug discovery programme, our company synthesises over a thousand novel compounds that are evaluate through our screening cascade, most of which is in vitro.

Only those compounds that pass stringent criteria are proposed for in vivo evaluation. In the final selection for proposed human use in clinical trial, each of these late stage-filtered compounds is likely to require multiple rounds of in vivo evaluation before nomination for

clinical trials is accepted by the authorities.

Sample sizes for our experiments are estimated from previous experiments and pilot studies. We employed statistic methods utilising typical variations observed in our own earlier experimentation to calculate the minimum numbers of mice to be used whilst ensuring that the results are statistically significant.

Most data are quantitative for which non-parametric statistical analyses are appropriate.

For our established models, we have determined the numbers of mice required to enable us to detect inhibitory effects of 20-30% (the minimum therapeutic effect considered to be of value). For superficial tumours, group sizes of 6-8 and for internal tumours and metastatic models, 8-12 are standard. This represents the number of animals used for one round of evaluation for one tested compound.

For the optimisation of new method of fibrosis induction, we have based our calculations on the published scientific data produced by utilising this method for in vivo research.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We used the NC3Rs' experimental design guidance to plan our experiments, practical steps, and statistical analysis. We utilised recommended methods of randomisation and blinding the studies. We also plan to use the appropriate statistical analysis methods that allow the statistically significant output with the minimal numbers of observations.

Experiments are designed using the principles in the experimental design tool on the NC3Rs website, using ARRIVE guidelines for reporting studies, LASA, Animals (Scientific Procedures) Act 1986 guidelines and AWERB advice.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

New therapeutic compounds to be evaluated in our project will have been first assessed in tissue culture (both simple tumour cell monolayers and more complex 3D functional assays) and triaged for potency, stability, and tolerability both in vitro and in other projects.

Failure at any of these stages, limits the number of compounds going forward for testing in animals. As far as possible we use cells in which we express luminescent or fluorescent markers that emit light, enabling detection of tumours inside mice using optical imaging, which is quick and requires only light anaesthesia. Previous experience and data from pilot experiments further allows us to optimise the number of animals necessary for realising the project.

We employ methods such as luminescence imaging to locate and follow the development of internal tumours and/or fibrosis and their response to therapy where possible. Thus, fewer mice are required, and studies can be terminated before the animals experience significant symptoms. We ensure that we obtain the maximum possible information from each tumour and tissue samples, assessing not only tumour growth, but also correlating efficacy with drug levels and biomarker responses to give statistically robust data in proof-of-concept trials.

Furthermore, in the case of pulmonary fibrosis we plan to utilise a novel method of delivery of fibrosis- inducing agent. This new method offers great improvement to other, widely used method of delivery, by providing homogenous distribution of the agent and reducing variability. This will allow us to further reduce the numbers of mice required for our studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are the least sentient species that are appropriate for in vivo drug development studies and are widely used for this purpose, due to their anatomical, physiological, and genetic similarity to humans. Advantages of rodents include their small size, ease of maintenance, short life cycle, and abundant genetic resources. Most of our work is carried out using well characterised human tumours, grown orthotopically as xenografts in congenitally immunodeficient adult mice to avoid tissue rejection.

Occasionally, to investigate effects of interventions in mice with a normally functioning immune system, well-characterised mouse tumour models, transgenic mice and primary transplants in syngeneic hosts may be used.

Mice are socially housed in ventilated cages, using sterile food and bedding. All procedures are carried out in laminar flow cabinets, in a manner that does not expose the rest of the group of animals to the procedure, to avoid any distress that it might cause. Mice are continuously monitored throughout the duration of studies and any signs of discomfort, pain, and/or stress will be immediately addressed, according to the guidelines and in close cooperation with NACWO and vet.

Suffering will be minimised by keeping tumour sizes within tolerable and acceptable limits and according to recognised guidelines. Compounds are delivered using previously determined well- tolerated doses and schedules and are generally of low toxicity. In most of the cases we will deliver these agents via oral gavage, to induce a rapid change in body response and at the same time to assess oral bioavailability of these potential drugs.

Why can’t you use animals that are less sentient?

Non-mammalian animals are limited in their use because their tissues, organs and immune system are too different from the human immune system to provide relevant results. We also can’t use embryos or very young animals as they are not fully developed and will not respond to our interventions in relevant way.

How will you refine the procedures you're using to minimise the welfare costs

(harms) for the animals?

The current 2 FTE highly experienced licensees for in vivo studies who work on this programme collectively, along with our other FTEs (Chemists and Biologists) have a breadth of experience in both classical cytotoxic drug discovery and novel molecular targeted agents; particularly important where combined therapies need to be assessed. In addition, several staff members have expertise in discovering new tumour models, in particular the advanced surgical skills necessary for implantation of tumours in ‘orthotopic’ sites and an understanding of patterns of metastasis.

Our animals will be monitored throughout the study, and the frequency of monitoring will be adjusted depending on the animal needs. For example, animals that lose 10% or more of body weight after the treatment will be monitored on a daily basis and placed on a special mash diet to help them regain the appropriate weight. If the animals in question are still losing weight and approach the limit of acceptable body weight loss, they will be humanely killed.

Tumours will be closely monitored for the size and the state. we will use a special scoring table that we have developed for a different project that will allow us to assess the condition of tumours with relevance to potential distress it may cause (e.g. ulceration).

These animals will be treated following advice of the vet and NACWO. The treatment may involve topical agents reducing inflammation, analgesia, with strict deadlines for improvement. If improvement is not observed, animals will be humanely killed to avoid further experiencing of discomfort.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will perform our experiments using published guidelines (PREPARE, NC3Rs guidelines) and the best practices established in our animal facility, according to the NC3Rs guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly check information on NC3Rs website, we also signed up to the NC3Rs newsletter. Furthermore, we will have regular meetings and reviews with the relevant informed people in our animal establishment to keep up with the latest guidance for 3Rs.

Additionally, every year we will participate in retrospective reviews organised by our animal establishment, where any current advances and methods that can help with implementation of 3Rs are discussed.

33. Cellular and molecular mechanisms in the development and persistence of pain associated with tissue injuries

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Pain, Tissue injury, Pain signalling mechanisms, Analgesics, Burn injury

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to identify molecules, which could be used as targets for new drugs to control pain that follows tissue injury and subsequent inflammation.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

When healthy human and animal body tissues are subjected to mechanical, thermal (heat or cold) or chemical impact, these impacts may result in pain and/or tissue injury (noxious impacts). This pain is essential for survival, and where no injury is caused, the pain stops

almost immediately after the impact source is removed, for example, the pain, induced by touching a hot surface, eases as soon as you move your hand away.

When injury is inflicted by the impact, the tissue damage requires healing. In this case the pain will normally ease only when the injury is near to fully healed. However, often this 'tissue injury-associated pain' remains even after healing and can be unbearably severe for the patient.

Currently available treatments used to control severe and persisting tissue injury-associated pain often fail to provide satisfactory pain relief. It is estimated that ~50% of people experience persisting pain at least once during lifetime (Hagen et al., Scand J Pain, 2020, 20: 357-589). It is also estimated that currently approved analgesics provide satisfactory pain relief on in a minority of patients suffering persistent pain (Jayakar et al., Sci Transl Med, 2021, 13: eabj9837). The lack of satisfactory control of severe and persistent pain leads to suffering and a series of physical and mental diseases. This ruins the quality of life of both patients and their relatives and imposes a significant demand on health and social services as well as a financial burden on the patient, family, economy, and society.

To improve patient care and reduce the burden inflicted by severe and persistent tissue injury- associated pain, new highly effective painkillers (analgesics) must be developed. This aim means that we must gain a better understanding how tissue injury-associated pain develops and persists.

What outputs do you think you will see at the end of this project?

Our work under the expiring project licence has led to a significant discovery regarding mechanisms (processes) involved in the development and persistence of one of the main types (‘modality’) of pain, “burning pain”, of tissue injury-associated pain. That discovery serves as a starting point for the work we will perform within the tenure of this license. Hence, we will identify mechanisms leading to the development and persistence of tissue injury-associated pain further. We expect to identify molecules, which are vital for severe and persisting pain that occurs following tissue injuries. Further, we expect that some newly-identified molecules will be suitable for the development of novel analgesics. We also expect that our discoveries will lead to high impact publications, depositing a series of important information in publicly available data repositories, filing patents and the development of strategies for developing new highly efficient analgesics.

Who or what will benefit from these outputs, and how?

In the short term, the main beneficiaries will be the researchers and wider scientific community through the gain of important theoretical and technical/technological knowledge and deepening the understanding of mechanisms involved in the development and persistence of pain associated with tissue injuries. Our data will also be used to “feed” various non-animal models, such as “conventional” computer models and those which involve artificial intelligence.

By the expiry of this project licence, we expect that the findings will lead to the development of novel strategies to generate highly effective analgesics. Hence, in the long term, through the development of novel analgesics, beneficiaries will include patients suffering from tissue injury-associated pain. Further beneficiaries will include patient’s

families, health and social services, economies, and societies. In the long term, researchers, the institution, and research support-providing bodies will benefit from the commercial value of the expected patents.

How will you look to maximise the outputs of this work?

We will publish all the data we generate either through open access publications or publicly available sources. These data will include all details of the approaches and all results whether they are positive or negative. I strongly believe that publishing negative data is as important as positive ones, to avoid repeating unnecessary experiments.

Some of the projects covered by the expiring licence are performed in collaboration with researchers in other universities and companies. We will continue and expand these collaborations to further increase our output.

We will also participate in public engagement to make the public aware of our data. If the findings are significant enough, a press release will be used to make the public aware of our activities.

Species and numbers of animals expected to be used

- Mice: 5025

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The project entails studying how pain develops and persists as a result of tissue injury. This involves complex pathways of communication between the injured site and the body's nervous system. In some cases, injury can result in significant changes in the structure of nerve cells that respond to our internal and external environment ('sensory neurones'). This is a complex and not well understood process, and although attempts have been made to replicate this in models that do not involve the use of animals, (e.g., through stem-cell derived sensory neurones), the reliability of the data collected has been low, and is not sufficient to compare to how this may take place in the human body. Due to the involvement of communications between multiple tissues (i.e. injured tissues such as skin, muscle, or bones, the immune system and nervous system) in the development and persistence of pain that follows tissue injury, reliable data how such pain develops and persists can be obtained only from in vivo models where those communications between various tissue are intact.

Pain is an adaptive response to tissue injury and subsequent inflammation to protect the injured tissues and help healing. Therefore, the cellular and molecular mechanisms involved in this adaptive response are near identical in human and other vertebrates, meaning that in vivo models of painful conditions in vertebrates largely recapitulate process in human.

By combining in vivo models and various methodologies including the control of specific genes in specific cells, we can dissect cells and molecules, which will serve as "targets" for new pain killers. The overwhelming majority of those methodologies are performed on neurones hence, those cannot be performed on humans.

Based on the reasons mentioned above, in vivo models offer a priceless source of information, which needed to improve human health. Therefore, to accomplish the goal of this project and to improve human health, using in vitro animal models is inevitable.

In this project, we will use mice. The overwhelming majority of biomedical research, which involves animals or animal tissues, uses mice. Therefore, a vast amount of data is available on mice and for comparing our results to previous data and future data by others, we will use mice.

Mice for laboratory research are available with various genetic backgrounds. These animals have thoroughly been characterised, and the suitability of each of those genetic backgrounds for various experimental purposes has been well established.

Further, the differences between mouse and human genomes have also been thoroughly studied. Thus, converting data obtained on mice into human treatment (‘translation’) is reasonably predictable. Also, controlled breeding of animals with distinct genetic backgrounds ensures a high degree of genetic similarity within colonies, which is important to generate reliable and reproducible data with high statistical power from as few animals as possible.

Human diseases must be modelled in animals for studying diseases develop and how they could be cured. Mice have been the preferred species to model various diseases, including tissue injury and inflammation. Mouse models are well-established and characterised, and they are highly reliable.

Controlling the function of genes is a highly valuable tool to find genetic mechanism needed for the development of diseases. Traditionally, mice are used in such experiments.

Although unsatisfactory control of severe, persistent tissue injury-associated pain in children is also an important unmet medical need, the majority of patients who require pain control are adults. Therefore, in this project, we will primarily use adult animals.

Typically, what will be done to an animal used in your project?

In a typical experiment, animal’s basal sensitivity to heat and mechanical stimuli are tested, then a model of inflammatory pain (surgery, injection) is generated. From post-surgical/post-injection day 1, sensitivity to heat and mechanical stimuli are assessed daily and interventions via daily injections to alleviate hypersensitivity is performed. A typical experiment lasts for 5 days. In a limited number of experiments pre-model creation interventions, such as injections or surgical interventions may be performed.

For studying the effects of injury in tissues and the resulting pain, it is necessary to (1) create models of inflammation and hypersensitivity in the mice and (2) test how injury changes sensitivity to thermal and mechanical stimuli through assessing “pain-related behaviour”. This latter assessment means measuring the reduction in the strength of thermal or mechanical stimulation which induces a withdrawal reflex reaction (i.e.

development of hypersensitivity). Assessing pain-related behaviour with thermal stimuli is done by projecting a light beam to an area of the body (typically one of the hind paws) and measuring the time needed to provoke a withdrawal reflex, a point during the stimulation when the animal experiences unpleasantness.

Assessing pain-related behaviour with mechanical stimuli is performed by pressing plastic filaments, which put pressure of various strengths to an area of the body (typically one of the hind paws). The strength of the pressure provoking a reflex withdrawal is then taken as the sensitivity. Testing “pain-related behaviour” will be performed in the majority of the animals repeatedly, as we have to record the baseline sensitivity before inducing tissue injury then to assess the effect of any treatment. Hence, typically, pain-related behaviour is measured for 5 times during the entire course of an experiment.

Models of tissue injury and inflammation are generated by surgery, or introducing substances that cause inflammation via injection, or replicating accidental injury such as burn injury. Model generation is performed once during the course of an experiment. However, other surgical procedures or injection (for example pre-treating animals) may also be performed during an experiment.

The location, size and duration of the tissue injury causing hypersensitivity is not expected to affect the animals’ normal behaviour such as feeding, grooming, or housing. Further, the hypersensitivity is not expected to induce spontaneous pain triggering pain-related behaviour such as licking or shaking the injured/inflamed area. Further, hypersensitivity to heat will not reach the level when pain is induced by temperatures below 38-39oC and mechanical hypersensitivity will not reach the level when a gentle touch provokes pain. The injured/inflamed area will not be in touch with anything during normal behaviour. Therefore, it will not be exposed to mechanical impacts, for example during moving around the cage, to induce pain.

Surgical interventions for inducing tissue injuries involve either:
- making a small incision on the animal's paw and underlaying muscle, then closing the wound with stitches, or
-making a small incision on the thigh, severing a nerve, which is needed to detect heat and mechanical impacts, and closing the wound with stitches.

The additional surgical intervention involves making a small skin incision to implant a device to deliver drugs, or to inject substances into a nerve before closing the wound with stiches. One of these interventions may be performed once during the experiments before generating the inflamamtory pain model.

All surgical procedures take no more than 15 minutes and are carried out under anaesthetic, using sterile surgical techniques. The site of incision is not expected to be in contact with the cage.

Inducing inflammation involves injecting a small volume of agents under the skin which is not expected to be in contact with the cage. Again, the hypersensitivity is not expected to reach the level of pain induced by innocuous stimuli ('allodynia').

Burn injury includes immersing one of the hind paws in 60oC water for 90 seconds under general anaesthesia. Animals are not allowed to gain consciousness during the induction of the injury and the pos-tinjury period, which lasts for a maximum of 3 hours.

Animals, after inducing the models survive for various periods (typically for 5-7 days).

Longer survival will be used only in limited cases when long term effects of treatment must be studies.

Either before or after inducing the pain model, animals may be treated with various drugs and agents. This involves injections, which starts, typically 1 day after surgery/injection. Treatment can be provided as prevention as well before generation the model. However, either pre or post-model treatment will be performed.

In a typical experiment, animal's basal sensitivity to heat and mechanical stimuli are tested, then a model of inflammatory pain (surgery, injection) is generated. From post-surgical/post-injection day 1, sensitivity to heat and mechanical stimuli are assessed daily and interventions via daily injections to alleviate hypersensitivity is performed. A typical experiment lasts for 5 days. In a limited number of experiments pre-model creation interventions, such as injections or surgical interventions may be performed.

At the end of the experiment, animals will be humanely killed and, typically, tissues collected for further molecular studies.

What are the expected impacts and/or adverse effects for the animals during your project?

Expected impacts include the development of a disease (injury or inflammation) which model human condition. This condition involves hypersensitivity of the tissues, swelling, and redness, which last from a few hours to a month. However, in the majority of cases the experiment will be terminated within a week. The hypersensitivity will not affecting animals' normal activities, such as feeding, drinking, grooming, social interactions, etc. The hypersensitivity will not induce spontaneous pain either (e.g. spontanous pain-related behaviour such as licking or shaking the injured/inflamed area), or reach the level when pain is induced by temperatures below 38-39oC or a gentle touch.

The severed nerve, in addition to serve a sensory function for the detection of mechanical and thermal impacts, also contributes to regulate the function of a few small muscles. Therefore, the nerve injury may also lead to temporal partial paralysis of those muscle, affecting using the limb to a small degree. However, this is expected to last only for a week.

The surgical procedures to implant devices (e.g. chips, device for continuous drug delivery) or injecting agents to peripheral nerves induce an inflammatory reaction, which is associated with hypersensitivity. Wound closure may break and the wound could be infected, though that is highly unlikely as all procedures are done in aseptic conditions. Implanted devices may induce unpleasantness affecting animal behaviour. Injections of peripheral nerves may induce neurological symptoms such as partial paralysis leading the altered pattern of using limbs. However, again, this is highly unlikely as known agents will be used.

We will use various drugs substances to modify cellular and molecular mechanisms of pain signal processing. Some of these might be novel with little in vivo data. These might induce adverse effects (weight loss, behaviour, neurological, gastrointestinal, circulatory,

etc).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

10% non-recovery
45% mild
25% moderate
20% sub-threshold

What will happen to animals used in this project?

- Killed
- Used in other projects
- Kept alive at a licensed establishment for non-regulated purposes or possible reuse

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In this project, we will study mechanisms that lead to the development and persistence of pain, characterised by hypersensitivity to heat and mechanical impacts, following peripheral tissue injury. Both the development and persistence of hypersensitivity are based on systemic events involving complex inflammatory and neuronal mechanisms. While some parts of those mechanisms can be studied using various cell and tissue preparations, for studying the development and persistence of hypersensitivities in their entire complexity, animal studies are needed.

Pain that develops and persists after injury and inflammation is an adaptive response to protect the injured tissue and help healing. That adaptive response is nearly identical in humans and vertebrates thus, in vitro studies can provide reliable data for translation. By combining in vitro models and various technologies, we can identify cells and molecules key for the development and persistence of pain and will serve as targets for new pain killers. Importantly, due to the key role of neurones in the development and persistence of pain, in the overwhelming majority of our studies, we employ those technologies on neurones. Those technologies cannot be employed on human neurones. Therefore, to accomplish our overall goal, using animals is inevitable.

In addition to discovery of ket cellular and molecular mechanisms, studies for translation also require animals, as other studies at present cannot be reliably provide the necessary information. However, technology is rapidly developing, and we will monitor the availability of technologies which allow replacing animals and animals' cells/tissues.

Which non-animal alternatives did you consider for use in this project?

I have continuously monitored publications on mechanisms in nociceptive processing to find new alternatives to replace animals in my work. In addition, I regularly check various websites for alternatives to animal experiments (e.g. NC3Rs; EURL ECVAM, The Animal Welfare Information Centre). In addition to the "traditional" in vitro approaches (e.g. heterologous expression of various molecules), and some very recent advances, which we have been using, no other approaches have emerged in recent years.

Using induced human pluripotent stem cell (iPSC)-derived cells and organoids using iPSC-derived cells are exciting approaches to replace a significant proportion of studies using laboratory animals. We have started using iPSC-derived sensory neurones and tested them for basic responses. For studying certain cellular responses, for example, composition and organisation of molecular networks, iPSC-derived sensory neurones are excellent, and we will use them.

In silico modelling is another alternative. We are planning to use this approach to elucidate how changes in gene expression, lead to persistent pain. We will also improve existing modelling by “feeding” the models with our own data.

Artificial intelligence (AI) is a rapidly developing technology, and it certainly can handle significantly higher amounts of data than human individuals. Hence, we consider using AI to predict molecular events involved in the development and persistence of pain associated with peripheral tissue injury/inflammation.

Why were they not suitable?

iPSC-derived organoids: Primary sensory neurones, the cells that provide innervation to our tissues (i.e. skin, bone, muscles, and various hollow organs), are pivotal for the development of pain associated with tissue injury; primary sensory neurones provide the information about noxious (and innocuous) stimuli, the presence of injury and inflammation. Further, they develop hypersensitivities to noxious and innocuous stimuli; hence, they are pivotal for the development and persistence of hyperalgesia and allodynia.

It has been known for decades that primary sensory neurones are heterologous.

Recent gene expression-based classifications indicate the presence of up to almost 20 types of sensory neurones. Our most recent data indicate a strong correlation between function and type of neurones (i.e. the presence of a high degree of specificity). Further, our recent findings also indicate that within the almost 20 main types of neurones, subtypes might be present. At present, we do not know the details of how primary sensory neurone types and sub-types develop, and what molecules at which time point are needed to induce the generation of various sub-types of neurones. Hence, current technologies do not allow us to induce specific groups of neurones. Hence, while the use of iPSC-derived cells is useful, the translational value of those data is limited at present.

In silico modelling: In silico modelling depends on available data, which can be used for generating the model. In theory, a model that includes the injured tissue – the immune system – sensory neurones communication in its entire complexity could be built. However, only a fraction of the data to build such a complex model is available.

For example, only a fraction of molecules released from the injured and inflamed tissues is

known and we do not know all those molecules which act on sensory neurones.

Further, we do not know all the cellular events triggered by interactions between injured and inflamed tissues and primary sensory neurones. Unfortunately, those huge gaps do not allow us at present to model the complexity of injured tissue – the immune system – sensory neurones communication and the resulting molecular events in primary sensory neurones. As mentioned, we will use our own data to improve existing models.

AI: Similarly to in silico modelling, AI also depends on available data. As discussed above, we have huge gaps in our knowledge regarding the molecular events in injured tissue – the immune system – sensory neurones communication. Therefore, AI may be used for certain predictions, but it cannot replace experiments conducted on laboratory animals. Similarly to in silico models, we will try to improve existing modelling with our own data.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the number of animals based on our previous experiences. Those experiences show that for behavioural studies 6-8 animals/group are needed to have meaningful data. For functional in vitro studies (electrophysiology, calcium imaging), cells/tissues from 3-6 animals/group are sufficient (depending on the type of investigation). Our experiences also show that for studying the expression of certain molecules we need tissues from 3-6 animals; however, in some cases, pooling is required. In those cases, we have to pool tissues from 2-3 animals to generate each replicate.

The calculation of animals is also based on our experimental plan. Hence, when using mice lacking or over-expressing certain genes for studying the effect of an injury, at least six groups (wild type and genetically modified, in each of those naïve, sham or injury) are needed. When studying the effect of a drug, we need at least 9 groups (naive, sham, injured; each without drug, vehicle, or drug with one dose).

Finally, the calculation uses our previous licence, when we predicted using 5800 animals (rat and mice together). In this project we will use only mice and less models than we used before. Therefore, we could reduce the number of animals.

We use NC3R's Experimental Design Assistant to finalise the number of animals we need for each set of experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We design all studies by following the principles of Good Laboratory Practice, ARRIVE guidelines, revised National Institutes of Health Guide for the Care and Use of Laboratory

Animals, the Directive 010/63/EU of the European Parliament and the Council on the Protection of Animals Used for Scientific Purposes and the guidelines of the Committee for Research and Ethical Issues of IASP published in PAIN (16 (1983) 109- 110). As mentioned above, we use NC3R's Experimental Design Assistant to establish the number of animals we need for each set of experiments.

Our design includes careful planning of using animals to avoid unnecessary repetitions. Hence, we make sure that each control group of animals is used as control for as many treatment groups as possible. Further, during the design, we make sure that we collect as much data as possible from each animal. Hence, the design includes assessing various aspects of the condition (e.g. pain-related behaviour, measuring oedema, temperature, and collecting all tissues, which might be used for assessing the expression of genes or proteins).

At present, surrogate markers of pain (e.g. increased expression of genes, gene products, post- translationally modified molecules) inform us about the activation of the nociceptive system. Studying the cell type expressing those markers is useful.

However, at present, markers do not inform us about changes in various modalities of pain (e.g. heat, cold, mechanical). Therefore, at present, we must use behaviour studies. However, advances in analysing molecular changes during the development and persistence of pain have accelerated. Therefore, we can expect a significant increase in surrogate markers-derived information that makes behaviour studies unnecessary. During our work, we follow relevant literature for more refined and reliable new models and biomarkers for pain in laboratory animals.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Breeding is tightly controlled. When we have enough animals, we stop breeding and resume only when needed. If we have to purchase animals, only the necessary number of animals is ordered. In this way we make sure that animals are not killed because they are not required for any of our experiments.

If needed, we will perform pilot studies to ascertain the effective dose and the development of any adverse effects of drugs which have not been tested thoroughly in vivo. These pilots, in addition to reducing animals, which could be administered ineffective doses of the drugs, will also significantly contribute to refinement.

We also share tissues from animals we use. Sharing includes “within the group” sharing when tissues of animals used for a particular study are given to people running another project in the group. We also offer tissues outside of our group.

There is a list of available tissues, which we share when we are approached.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The aim of this project is to elucidate mechanisms involved in the development and persistence of pain associated with peripheral tissue injuries. Hence, it is inevitable to use models that lead to the development and persistence of some discomfort in the form of hypersensitivity. However, all the models we use on conscious animals are associated with mild or moderate severity. When the severity level is expected to exceed the moderate level (for example, in burn injury), animals are kept under general anaesthesia, and they are not allowed to regain consciousness at any point in the procedure. Further, if short-term models are used for other reasons than assessing hypersensitivity or long-term cellular or molecular changes, the procedure is also done under general anaesthesia. Finally, general anaesthesia will be used during the generation of each model, unless an assessment of immediate behavioural changes is required (e.g. “formalin test”). However, only a limited number of animals <5%) will be used in such tests.

The severity level of all models, except the burn injury model, is moderate. Hence, the hypersensitivity will not affect the animals' normal feeding, grooming and social behaviour. Animals are not expected to experience spontaneous pain, and gentle touch or temperature below 38-39oC will not induce pain.

Conscious animals will have moderate and short discomfort when hypersentivity is assessed.

MODELS

Somatic tissue injury models

Tissue injuries are either due to accidents, medical interventions (i.e. surgery) or degenerative diseases. In the project, we will use models which can replicate events occurring in humans in these types of injuries as reliably as possible.

Incision: The “paw incision” model post-operative inflammation, tissue healing and pain. This model replicates events occurring in human postoperative pain, which is one of the most frequent sources of peripheral tissue injury-associated pain. The severity level of this model is moderate.

Burn: Burn injury is one of the most frequent accidental tissue injuries, which is associated with excruciating pain. Animals in this model are under general anaesthesia during the entire procedure, they are not allowed to gain consciousness at any point of the procedure. This type of burn injury allows assessment of cellular and molecular events occurring within a few minutes - three hours after the injury.

Other models involve assessments for several days, which require the administration of analgesics, which is highly likely to lead to false discoveries.
Somatic inflammatory models

All types of tissue injuries, are followed by inflammatory reactions therefore, inflammatory models are widely used to replicate events following tissue injuries.

Carrageenan: Carrageenan, when injected under the skin (subcutaneously), leads to local inflammatory reactions and hypersensitivity to mechanical and heat impacts. The hypersensitivity fades away within 5 days. This model is usually used to model acute inflammatory reactions.

Complete Freund's Adjuvant (CFA): CFA, when applied by a subcutaneouseous injection, leads to an inflammatory reaction that lasts up to 30 days. The CFA-induced inflammation has a slower development than the carrageenan-induced reaction, and it lasts for a significantly longer time, up to 30 days. Therefore, the CFA model is used to mimic chronic inflammatory reactions.

Irritant models: We are applying those agents, identified in injured/inflamed tissues either topically or via injection into various tissues (for example, subcutaneously in the paw, flank, cheek, etc.) and assess how they induce pain and inflammatory reactions (e.g. oedema) or other physiological/pathological responses (e.g. body temperature) for a few hours.

In addition to the newly identified agents, we also use known activators (e.g. capsaicin, formalin, mustard oil, etc) of the putative target molecules as control.

The majority of the injections will be performed under anaesthesia. Only when absolutely necessary due to expected immediate effects (<5%) will the injection be administered to conscious animals. The substances we will inject will not lead to any lasting harm.

Nerve injury model
Tissue injuries often include injury to peripheral sensory nerve fibres (peripheral sensory neuropathy, PSN). PSN is also associated with hypersensitivity of tissues to thermal and mechanical stimuli.

Therefore, we will also use nerve injury models to elucidate cellular and molecular mechanisms of tissue injury-associated pain.

Spared nerve injury model: The spared nerve injury is the least invasive nerve injury model. The resulting pain is restricted to a small area of the paw.

METHODS
Pain-related behaviour
In order to find whether or not the model is generated successfully, and interventions have any effect, we must quantify hypersensitivity.

Using the Hargreaves apparatus and the thermal probe to assess heat hypersensitivity and von Frey filament to assess mechanical hypersensitivity are the most widely used and most refined ways that provide quantitative data characterising hypersensitivity. It is of high importance to note that animals experience short-lived maximum moderate levels of pain as when they experience an unpleasant temperature or mechanical pressure, they simply remove the affected area from the heat source.

Pharmacology

In order to find the function of a gene product or to show that controlling a certain gene product, leads to analgesia, we have to use in vivo pharmacology. Compounds are applied by injection through subcutaneous, intraperitoneal or intrathecal routes. When needed (e.g. intrathecal injection) the applications will be done under general anaesthesia (AB).

During the interventions, we will primarily use compounds characterised in previous in vivo experiments. If such drugs are not available, either we will use a genetic approach (see below) or will conduct a small-scale pilot study using non-recovery anaesthesia. Only compounds that do not have toxic or adverse effects will be taken forward to animals with or without general anaesthesia.

All compounds will be sterile and applied to disinfected areas. Further, we will follow guidelines in injection volumes (please see Morton et al. Lab Anim. 2001 35: 1-41, PMID: 11201285).

Neuronal degeneration/desensitisation
To find the function of certain neurones, it is important to block their activity. In some circumstances it can be done only by desensitising or eliminating them. Thus, we will use various drugs, toxins or genetic agents injected either into peripheral nerves (for example sciatic nerve), intrathecally, or peripheral tissues. This approach does not require major surgical interventions or involve effects, which prevent animals' normal behaviour. Effects in these models never exceed moderate severity levels.

Controlling gene expression
Controlling gene expression is one of the most efficient and refined ways of elucidating pathological processes as well as assessing the therapeutic effects of targeting certain molecules. Hence, controlling gene expression has a high translational potential. Therefore, we will use various genetic models in our work. It is important to note that we do not expect any harmful effects of controlling gene expression. The only effect we expect is reduced discomfort/pain of animals in the various models when the stimulus is applied.

With using various breeding startegies, we will use global deletion or condition deletion of specified genes. In addition, conditional over-expression will be used to obtain additional evidence for the function of a gene. We will use this approach by injecting viral particles carrying genetic materials (e.g. cDNA of the desired gene(s)) into various tissues.

Animals with gene deletion or insertion will be purchased and then bred for our studies.
Implanting devices
Implanting various devices (microchips, osmotic pumps) constitutes a major refinement. By using microchips, we can read various measures of physiological/pathological functions precisely without disturbing the normal behaviour of the animals.

Applying various drugs by injection is associated with distress, even if that is given under anaesthesia. In some cases, multiple injections are needed, which would increase distress. By implanting osmotic pumps, which deliver compounds continuously, we can reduce the time when animals' normal behaviour must be disturbed. Further, osmotic pumps provide constant concentrations of compounds at a given area, which leads to more reliable data and potential reduction in the number of animals needed for a given study.

Why can’t you use animals that are less sentient?

Each animal has functions, which provide warning for imminent dangers and help recovery. Due to some conserved evolutionary processes, certain aspects of cellular connections, functions, and genes can be assessed in non-vertebrates. However, vertebrates, particularly mammals exhibit pain-related behaviours, which have the desired translational value.

The development and persistence of pain associated with tissue injury and the subsequent inflammatory process involve highly complex mechanisms via the immune and nervous systems. Further, while pain associated with tissue injury develops in children, the majority of patients are adults. Both the immune and nervous systems must reach a certain maturation point when they can work together and perform pain signalling functions. However, from that point of maturation, immature animals also exhibit pain-related behaviour. Further, maturation of both the immune and nervous systems continue well beyond birth; hence, pain signalling processes identified in embryos and foetuses significantly differ from those in adults.

We use conscious animals only when it is absolutely necessary. Thus, we use conscious animals for behaviour studies and models that require a longer time to develop (e.g. surgical models and some inflammatory models). However, even in those cases, we keep animals for the least time necessary for the study. In all other cases we perform the experiment under general anaesthesia and use surrogate markers for pain.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

During this project, we aim to elucidate mechanisms involved in the development and persistence of pain associated with tissue injury. Hence, modelling painful conditions is inevitable. However, we are using models, which, while providing meaningful data, induce the least harm to animals. These models are generated using approved and refined procedures.

All agents we use are prepared using sterile techniques, and injection and surgical sites are cleaned and sterilised. All procedures are done under aseptic conditions. Surgical interventions and injections are done using appropriate and approved techniques at carefully chosen sites of the body. The injection volume is always chosen appropriately using guidelines from, e.g. Morton et al. 2001.
Animals are left for acclimatisation for a week after arrival before any procedure.

Further, following any procedure, animals are monitored closely for signs of complications and adverse effects. If complications develop, they are promptly treated according to the guidance from NVS. Animals which do not respond favourably when complications are treated are humanely killed. Adverse effects are promptly recorded and reported, and if necessary, animals are humanely killed. Importantly, the severity level never exceeds a moderate level in any of the models or procedures we use on conscious animals.

The models we use are models of tissue injury and inflammation. Those models are inevitably associated with the development of hypersensitivities. In order to find mechanisms that underpin the development and persistence of those hypersensitivities, we have to assess and quantify them. To get meaningful information, we cannot use analgesics as they would cover up important mechanisms and we would reach false

conclusions. However, when surgery is needed to inject a peripheral nerve or implant a device, analgesia (e.g. carprofen in drinking water) will be administered. In addition, following all interventions, animals will be closely monitored. Following paw incision or spared nerve injury, animals will be monitored at least every 3 hours during the day of the surgery up to 10 pm. After the first day, they will be monitored daily. After injecting substances into the paw, again, animals will be monitored daily. Monitoring will include gait, grimace scoring, and weighting if needed. Animals will also be monitored for exhibiting any humane endpoints. If an animal exhibits any sign that exceeds the expected one or any humane endpoint, it will be humanely killed.

Assessing pain-related behaviour as a withdrawal reflex is the most frequently used approach to assess the development and persistence of pain. The approach is based on hypersensitivity that characterises pain in tissue injury. During the measurements, we stop using stimuli stronger than the weakest that induce the withdrawal reflex. That stimulus is not expected to induce pain, it rather induces an unpleasant experience which lasts until the animal removes the exposed tissues.

Animals are always free to remove the exposed tissues. Importantly, we never use stimuli which could cause tissue damage or make existing injuries more serious.

Some models need several days to develop; hence, it is not feasible to keep animals under anaesthesia. Nevertheless, we keep the time at a minimum, assess behaviour and terminate the experiment when hypersensitivity reaches the peak. Endpoints are rigorously defined, and we observe them to ensure that severity levels never exceed moderate levels.

All procedures will be done by highly competent researchers. All researchers will be doing procedures under supervision until they reach a highly competent level.

Procedures will be planned to make sure that daily checks (several times if necessary) can be done.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will adhere to ARRIVE guidelines. Further, we will follow NC3Rs and LASA guidelines, and published protocols (e.g. Morton et al. 2001 for the administration of substances) in our experiments and carefully consider introducing developments published by other laboratories.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I regularly visit NC3Rs' website to read news and articles on NC3Rs Gateway. I have subscribed NC3Rs' newsletter and read it regularly. I regularly communicate with colleagues studying various aspects of biomedicine, and those communications involve experiments on animals, welfare and 3R issues, particularly how to reduce the number of animals and refine models. I am also in regular contact with NACWOs, training and competency assessors, and NVS. Further, I participate at relevant symposia and /webinars, reading relevant articles on the NC3Rs Gateway.

34. Reagent Production in Support of Diagnostic Tests

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Reagent, Antigens, Antisera, Production, Blood

Animal types	Life stages
Rabbits	adult
Domestic fowl (Gallus gallus domesticus)	neonate, juvenile, adult
Turkeys	juvenile, adult
Guinea pigs	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The objective of this project is to produce reagents for either direct use in diagnostic tests or the improvement of them.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The tests used or developed as a result of this project support disease diagnosis and improvement of health of farmed animals. Some of the tests also cover zoonotic organisms such as E.coli, Salmonella and Lyssaviruses which can have significant impact on human

health.

The Unit producing these reagents is responsible for producing approximately 400 different diagnostic reagents. Approximately 60% of these reagents are polyclonal antibodies, 11% are antigens and the remainder are kits and other reagents. This licence supports production of approx. 160 of these reagents.

What outputs do you think you will see at the end of this project?

The ongoing availability of high quality diagnostic reagents will be maintained to help ensure the work of both animal and human health and animal welfare through the Organisation's national diagnostic capacity for disease diagnosis and import/export testing in order to control diseases that are exotic to the UK. The output is essentially that there is not interuption in this capability.

For example, the antibodies against different salmonella serotypes will allow epidemiological investigation of disease outbreak, preventative action and indirectly support publications and other communications.

Who or what will benefit from these outputs, and how?

The work of this project is ongoing benefits potentially realised as each antibody, antigen or blood is delivered to the organisation's national diagnostic capacity for disease diagnosis and import/export testing in order to control diseases that are exotic to the UK.

The benefits of accurate and timely diagnosis of infectious disease are potential treatment/or euthanasia of animals both of these techniques prevent or reduction in spread of the disease. Import and export testing takes to a national level, of maintaining disease freedom and prevention of the spread of disease. These benefits go across animal welfare and economy, including ability to trade internationally.

A large amount of the project licence covers generation of antibodies for zoonotic diseases such as salmonella. The benefit of this is minimising the impact of outbreaks in humans as the tests using these antibodies to identify serotypes contributes to the epidemiological investigation and monitoring of the on farm situation. This understanding is also important in helping understand the development and spread of antimicrobial resistance in the salmonella, but this due to its epidemiological research nature is longer to achieve.

Reagents are also sold commercially for similar for a wider realisation of benefits.

How will you look to maximise the outputs of this work?

This is a service licence with production of these biological reagents is carefully linked to demand for diagnostic use and to supply research areas with bespoke reagents.

Species and numbers of animals expected to be used

- Guinea pigs: 150
- Rabbits: 1000
- Domestic fowl (Gallus gallus domesticus): 560

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The animals selected have been proven to be the best model to produce the material of the best specificity and sensitivity for testing e.g. chickens for Marek's Disease antibody, Swine flu and Salmonella pullorum, rabbits for E.coli and salmonella typing sera. To produce antisera the animal has to have competent immune system, this varies from starting with day old chicks (for the Marek's Disease antisera) to rabbits that are group-housed and a few weeks old. In some cases the organisations such as the World Organisation Animal Health (WOAH) dictate a particular model should be used.

Marek's disease antigen production is done in chickens as this is natural host for the disease, with the experiment starting using chicks that are approximately two weeks old.

Swine flu antiserum which is raised in chickens as part of refinement as the volume of serum required is sufficient in chickens, is suitable for its diagnostic purpose and prevents use of a pig.

Guinea pig blood is required for porcine parvovirus (PPV) and avian testing using haemagglutination inhibition test (HAIT) and this blood has been validated for use with these tests. There have been delayed deliveries from commercial suppliers from Europe and there's no way of predicting whether the blood will arrive fit for purpose until we start testing. If blood cells have been delayed in delivery this can cause the blood cells to lead to a test failure. This means that the guinea pig used to provide the blood will have been culled without any benefit. Test failures lead to a waste in reagents, consumables, time and needless loss of guinea-pigs on these occasions. Delayed deliveries have caused multiple submissions to exceed test turn around times whilst waiting several weeks for a suitable batch to arrive.

Adult ex breeder guinea pigs are used to provide blood. This is considered ethical use of animals as they would be otherwise be culled at the end of their breeding life and discarded. Adult animals also provide a higher yield of blood which means that half the number of animals are required compared to use of younger animals.

Typically, what will be done to an animal used in your project?

Protocol 1 Polyclonal antiserum production in rabbits (primarily against E coli and Salmonella)
Inoculation programmes vary with type of antibody required to be produced using inactivated antigen.
There are 2 programmes involving up to 6 intravenous inoculations. A third programme that uses adjuvant, consists of up to 4 inoculations via subcutaneous and intramuscular routes. All programmes will end with exsanguination under terminal anaesthesia.

Protocol 2 Production of Marek's Disease Virus Antisera in chickens

Intra-muscular (IM) inoculation of 1 day old chicks with live Marek's Disease vaccine - followed by 4 IM boosts with small amounts of Marek's Disease virus. After 90 days the birds will be exsanguinated under terminal anaesthesia.

Protocol 3 Production of Marek's antigen in chickens
Chickens will be infected by intra-abdominal injection with a live vaccine strain of Marek's disease virus at a minimum of two weeks old. They will be euthanased by schedule 1 method when the feather follicles swell, indicating suitable antigen harvest.

Protocol 4 Non-infectious production of Antibodies to other diseases using chickens
Chickens will be inoculated using inactivated antigen by the intra-muscular route (each breast). Birds will be between 6 - 12 weeks old when inoculated. Non ulcerative adjuvant (Montanide) will be used for the first inoculation only, up to 3 further inoculations may be required. Swine flu antiserum production will require test bleeds to establish the required titre is present before culling.It may be necessary to produce antiserum against other diseases in chickens using an inactivated antigen in the future.
At the end of the study birds will be exsanguinated under terminal anaesthesia.

Protocol 5: Guinea pig blood donor
Blood from healthy Guinea pigs is routinely required for production of red blood cells for use in the haemagglutination inhibition test (HAIT) test. Whole blood is collected via heart bleeding under terminal anaesthesia. Guinea pigs will be killed by a Schedule 1 method (overdose of anaesthetic) after exsanguination.

What are the expected impacts and/or adverse effects for the animals during your project?

Protocol 1. As the inocula have undergone inactivation procedures, no adverse effects in terms of systemic disease are anticipated. With the protocol using adjuvants there may some localised swelling, lumps or inflammation around the injection site.

Protocol 2. An attenuated commercial Marek’s Disease vaccine is used for the first inoculation, this is followed by inoculations of small amounts of Marek's Disease virus for the rest of the inoculations, no adverse reactions were observed in the previous studies in 2019 or earlier in 2018.

Protocol 3. Adverse effects expected to be limited to lethargy and inappetence and swelling of the feather follicles. If this persists more than 24 hours the birds will be euthanased. No adverse reactions were observed in the previous studies in 2020.

Protocol 4. As the inocula have undergone inactivation procedures, no systemic adverse effects are anticipated. With the protocol using adjuvants there may some localised swelling or inflammation around the injection site.

Protocol 5. As the guinea pigs are used as blood donors only and will be anaesthetised and terminally bleed, no adverse reactions are expected.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per

animal type)?

Rabbit - all mild severity
Chicken - mild severity for antisera raising, mild for the MDV antigen raising.
Guinea Pigs - non-recovery

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

A search of commercial suppliers and academic literature was undertaken for animal free antibody alternatives to the production of polyclonal antibodies in the areas of diagnostics that this licence produces antibodies for. Nothing suitable was found but this process will repeated for antibodies to each antigen requested.

The scientific reason for the lack of availability is that polyclonal antibodies contain a heterologous mixture of immunoglobulins against the whole antigen that the test is designed to recognise, whereas the other type of antibody, monoclonal antibodies, are composed of a single immunoglobulins against one epitope (a small part of the antigen). Whilst monoclonals lend themselves better to animal free production, the search done showed that polyclonal antibodies using in-vitro systems are not available or potentially possible for this application. However, animal free antibody techniques will continue to be investigated during the life time of this licence.

Currently theoretically what could be produced is mixture of monoclonals (a oligoclonal) this is because polyclonals are only being able to be produced in animals due to the complexity of the animal’s immune system. Antibodies used in the diagnosis of disease in animals also require high sensitivity and specificity and have to be validated, as any replacement would have to meet the same criteria as the current animal derived antibodies before it can be substituted.

An alternative approach is being undertaken on replacement of salmonella serotyping using antibodies, by the employment of genetic sequencing techniques.

This technique is being validated against various antibodies produced by this licence (and it predecessors).

This licence also raises antibodies in chickens against different diseases, primarily viral disease of chickens and similar approaches to replacement are being taken.

This licence is structured so as demand for animal derived antibodies produced by it reduces so will the production of antisera in animals.

Antigens (as opposed to antibodies) are also used in the diagnosis of disease. The requirement of this licence is to produce Marek's disease antigen in chickens. The reagent production unit invested a lot of time to perform trials using in-vitro (cell culture) techniques but this was not successful. There are commercial reagents available for Mareks diagnostic testing which are produced in-vitro, however these have poor specificity, rendering the test reagents not fit for purpose. There will be on going check for the availability of non-animal derived Mareks disease antigen of suitable quality during the lifetime of this licence.

Due to nature of the test , haemagglutination inhibition, there are currently are no alternatives to use of fresh guinea pig blood in the HAIT (Haemagglutination Inhibition Test) for tests for such pigs diseases as Porcine parvovirus.

Which non-animal alternatives did you consider for use in this project?

Whole genome sequencing is already used by the institute. Although it's use is expanding, routine diagnostics require the ability to undertake large numbers of samples with a short turn around time, which can only currently be provided by antibody technology.

Phage generated non-animal antibody generation were considered but rejected for reasons given in the next section and previous sections.

The use of non-animal alternatives will be reconsidered as the study progresses via exploring new scientific data, engaging with companies developing in vitro methods, consulting 3Rs centres and in- house expertise.

The establishment is committed to use alternative technologies to replace antisera where possible using non-animal derived antibodies and other techniques.

Marek's disease antigen production in cell culture was trialled but was not successful.

Why were they not suitable?

Animal free antibody (AFA) production techniques are not well established. A polyclonal antiserum (with possibly >100 different monoclonal Abs making this up) is currently not replaceable by animal free technology technology and it is not foreseeable when oligoclonal Ab mixtures will be accepted in diagnostic assay validation. This means that non-animal derived antibodies are not considered suitable at this moment.

However, this AFA approach is a significant part of the institutes replacement strategy and there are plans to progress this via a 3 year PhD project. This will potentially enable the technology going forward if it can pass the subsequent specificity and sensitivity requirements of diagnostic testing.

Marek's disease antigen production in cell culture was not suitable as we unable to achieve the required specificity and sensitivity.

Reduction

Explain how the numbers of animals for this project were determined. Describe

steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals required is based on the level of reagent demand which is monitored using an Integra stock management system enabling production to be planned so that minimal waste occurs and optimum stock levels are maintained. Additionally close contact is maintained with customers to ensure that reagents are supplied to them in appropriately sized volumes to minimise wastage. As a result the number of animals used is the minimum required to produce a 3 year batch to support testing requirements.

Where possible commercially available reagents are bought in e.g. various Indirect fluorescence antibody test (IFAT) slides and Salmonella Pullorum antiserum have been recently identified and validated for use, meaning we do not need to use animals to produce these in-house. Where we need to produce antiserum in house, a summary of searches from commercial sources are recorded on applications for approval for the AWERB committee.

The number of guinea pigs required is based on the number of tests carried out by the diagnostic lab, which is 1 test per week and the blood harvest from 1 guinea pig will be suitable for 2 tests over 2 weeks.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

For both antisera and antigen production the key to reducing numbers is to maximise the amount and titre of the material collected from each animal. The outputs of each batch are monitored and changes made to optimise the protocols if required. For example in the last licence the use of killed Salmonella inoculum with the addition of Montanide adjuvant raised the titres of the ~40 factors used in the A-S screening serum. This has meant that the final product can be diluted 100% and resulted in a significant reduction in the number of rabbits used from 72 to 36 per year.

Where possible pooled antigens are used as inoculum which reduces the amount of animals compared to raising the antibodies individually.

The amount of guinea pigs required is determined by the need to have fresh whole blood available on a weekly basis so that diagnostic tests can be supported as is required by Lab Testing at the organisation. We have established that guinea pig blood is viable for a two week period, this has enabled us to half the number of guinea pigs originally requested.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

These reagents are also supplied to several research groups within the agency therefore minimising the numbers of animals required.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

For antibody production rabbits and chickens are used predominantly as they have a proven ability to generate good immunological responses with high specificity of the resulting antibodies. This means there is less cross reactivity, so more accurate tests. Methods recommended by the World Health Organisation (WHO) guidelines are followed for raising Salmonella antibodies. Some protocols use adjuvant (non ulcerative) to enhance antibody titres. New adjuvants are being assessed with a view to improving titres and the animal experience.

For Mareks antigen and antibody production the inocula have been attenuated and therefore does not cause clinical signs.

The use of guinea pig blood in the HAIT is necessary to run the test and this blood has been validated for use with these tests. As the guinea pigs will be anaesthetised and terminally bled, any pain or suffering is minimised.

For both rabbits and guinea pigs, the use of enrichment (vegetables, tunnels, ice lollies etc) and more recently staff spending more time in the pens has shown that it has a calming effect on the animals.

All animals are killed at the end of each study.

Why can't you use animals that are less sentient?

To produce antisera a competent immune system is required, this involves using fully developed animals with associated sentience. The process takes several weeks and terminal anaesthesia is used when the animals are bleed out, which stops sentience and maximises yield of blood/antisera.

For antigen production live sentient animals are used only when cell culture or embryonated eggs cannot make antigen of suitable quality to give tests suitable specificity or sensitivity.

Guinea pig blood has been used in the HAIT test that has been validated and shown to be fit for purpose; there is no known alternative. The quantity required per week is 10-20 ml which matches the volume obtainable from a single guinea pig.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

As well as pre-start meetings involving the NVS, NACWO and animal care staff to ensure current knowledge is brought to bear, all projects are followed up by a wash up meeting. All aspects are discussed, was the project a success, what went well and if was anything that could be done better. If there are any suggestions for refining the procedure they will be considered and if appropriate, incorporated into the protocol. Frequent contact and communication with animal welfare staff is held throughout the studies, this helps to ensure up to date information is exchanged to ensure animal welfare is prioritised.

A refinement was implemented to the Swine Influenza antibody production in the chickens protocol. The number of inoculations and test bleeds involved have been reduced. A review of previous studies was carried out and showed that a 4th inoculation was not required as suitable titres can be achieved after a maximum of 3 inoculations. Reducing the amount of inoculations from 4 down to 3 will enable us to also remove one of the test bleeds.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

HO The Harm–Benefit Analysis Process HO Guidance to ASPA
HO Code of practice
World Organisation for Animal Health (WOAH) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.
RSPCA Guidance on Welfare of rabbits and chickens LASA Guidelines on substance administration NC3Rs web site
EURL ECVAM promotes and facilitates the use of non-animal methods in testing and research Animals in Science - Report on replacements to the use of living animals for antibody production

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I have regular contact with the NVS, NACWO, NIO and Lab testing through various forums and use of the library function which can scan for relevant publications. In developing the Marek’s Disease work, I have been in contact with researchers outside of the organisation who specialise in Marek’s Disease work.

Additionally, regular updates from the NC3Rs webpage (https://www.nc3rs.org.uk/) as well as keeping up to date with other accessible sources of information with advances in the field.

35. Mechanisms of tissue resolution and repair in inflammatory diseases

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Arthritis, Inflammation, Resolution, Pharmacology, Repair

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to uncover mechanisms leading to inflammatory joint disease and its resolution and to identify novel therapeutic targets.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Musculo-skeletal (MSK) disease remains a leading cause of morbidity and mortality. In the UK around one third of the population (over 20 million people) have a musculo-skeletal

condition and this includes people of all ages. 450,000 people in the UK have a diagnosis of rheumatoid arthritis and 10 million people have osteoarthritis. The pain, fatigue and lack of mobility associated with these conditions, leads to a significant economic impact with the cost of lost working days due to arthritis estimated to reach £3.43 million by 2030.

These conditions are long-term and currently incurable. Around 40% of rheumatoid arthritis patients do not achieve remission with current therapeutic options and there are currently no licensed drugs that can modify the disease process in OA, with current therapeutics only providing modest responses.

Animal models of arthritis have been crucial in furthering our understanding of the pathogenesis of arthritis and the development/testing of therapeutics.

What outputs do you think you will see at the end of this project?

Over the life of the project we will generate new scientific knowledge on the mechanisms driving the initiation and chronicity of arthritis. Data will be submitted for publication in open-access peer-reviewed scientific journals and presented at national and international meetings to share our research with the scientific community in a timely manner. We also regularly write methodological papers to share our practices with the scientific community and openly collaborate and train scientists from other groups.

Ultimately, we envisage that this project will lead to the generation of novel therapeutic approaches for treatment of rheumatoid and osteoarthritis.

Who or what will benefit from these outputs, and how?

In the short term (i.e. the lifetime of the PPL) the major beneficiaries will be the scientific community through publication/presentation of our work. We have a strong publication record (>30 peer-reviewed articles from the previous PPL of our group) as evidence of our commitment to this goal. In the longer term we hope that patients with arthritis will benefit from new knowledge gains leading to changes in therapeutics.

How will you look to maximise the outputs of this work?

We collaborate widely with other researchers, both national and international and will continue to share our practices and data. All data will be made openly accessible upon publication in line with policies of our funders, university and open access journals.

Our key method of disseminating our work will be through publication in peer-reviewed open access journals. We also undertake public engagement activities (e.g. Science festivals and visits to schools). We also regularly promote our work on relevant social media channels such as X and LinkedIn.

We share our genetically altered animals with the scientific community to facilitate the research of other groups around the world working in similar fields.

Species and numbers of animals expected to be used

- Mice: 7500

- Rats: 100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Juvenile and adult mice are being used for these studies as they are most appropriate and genetically amenable species to mimic the biological processes that drive inflammation and joint disease such as arthritis. The mouse models that we will use have been extensively characterised and are known to share many of the features of arthritis in humans. We perform significant amounts of in vitro and ex vivo research and have funding to develop an organ-on-a-chip model of the joint. This is still being optimised and is currently not able to serve as a replacement for studies in mice. The vast majority of the studies will be performed in adult mice, but we will also study juvenile mice in our spontaneous model of arthritis as this will give us an insight into the mechanisms that drive initiation of disease.

Typically, what will be done to an animal used in your project?

Typically, mice will receive an injection that will induce inflammation, in specific cavities like the peritoneum, or within the paws of the mice. In one of our models mice spontaneously develop joint inflammation at 4-6 weeks of age. We will monitor the inflammation in the joints by observing the paws as well as measuring the degree of swelling. We are interested in developing novel therapeutics for treating joint inflammation so mice will typically receive injections of drugs, which may be given daily or weekly. Typical experiments last 1-2 weeks for our acute models of joint inflammation and 3-4 months for the chronic model of joint inflammation.

What are the expected impacts and/or adverse effects for the animals during your project?

We maintain a colony of mice that are genetically altered to spontaneously develop a polyarthritis at around 4-6 weeks of age. This results in swelling in all paws and associated mild to moderate pain. The swelling is typically not such that it impacts mobility of the mice or their ability to feed. In all of our models of arthritis, mice will experience some initial weight loss, this is usually acute and the mice begin to gain weight again after this initial loss. We will take steps to minimise the stress and discomfort for arthritic mice by providing soft bedding and mashed food in containers on the floor of the cage to facilitate easy access.

The majority of the genetically modified mice that will be bred will not display any harmful characteristics basally. The exception to this is the non-obese diabetic mice, which become diabetic after approximately 18 weeks for females and 24 weeks for males. We do not typically maintain these mice to this age to avoid the induction of diabetes.

Administration of agents to induce inflammation and therapeutic administration of drugs involves various routes which carries a small risk of infection/inflammation at the injection site or an adverse effect on the respiratory system if administered orally.

It is important to emphasise that our approach to therapeutic innovation evolves around harnessing natural mediators that are found within the body that are tissue-protective, very much different from current anti-inflammatory medicines, so we do not envisage them to have side effects such as immunosuppression and associated susceptibility to infection. Minimally effective doses and treatment regimes will always be followed by fully trained staff. Animals will always be monitored after and during treatment to allow early identification and appropriate intervention should any adverse effects occur.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We do not expect any of the protocols to exceed moderate severity.

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Given the complexity of chronic inflammatory diseases it is not possible to fully understand the mechanistic aspects of disease progression and the effectiveness of novel therapeutics when solely relying on studies on cells/tissues grown in the laboratory. Animal models are required to dissect the complex interplay between different cell types and within distant organs that we observe in the pathologies we are studying (an example of this is our current study investigating heart problems in patients with rheumatoid arthritis).

Importantly, many of the pathways we are studying are conserved between animals and humans making our findings widely applicable. Furthermore, the assessments that we make in our models are based on clinically relevant parameters thus increasing the translational impact of our research.

Which non-animal alternatives did you consider for use in this project?

A significant component of our work involves the use of primary cells isolated from healthy human volunteers as well as patients and we have a significant track record of producing data using systems such as the 'flow chamber', which mimics white blood cell interactions within the blood vessels. We also routinely perform studies on individual cell types that are found within the joint (cells that are found within the joint lining called fibroblasts and macrophages, newly formed blood vessel cells as well as cartilage cells) as well as in co-culture models to assess for example, efficacy and toxicity of potential therapeutics prior to progressing to animal studies. We have adapted these models to take advantage of cells

and tissues from animals to facilitate the use of ex vivo studies where possible rather than in vivo experiments and we can successfully isolate and culture cells from arthritic joints of mice, such as fibroblasts. We are also in the process of developing organ-on-a-chip models of the joint using both human or murine cells and this strategy forms a major aspect of recently acquired funding from Versus Arthritis. In addition, as part of the Centre for Predicitve in vitro models (https://www.cpm.qmul.ac.uk), we have recently recieved funding from the EPSRC to fund 5 cohorts of PhD studentships from 2025- 2031 for organ on a chip development. We plan to develop new in vitro models of arthritis and also to connect joint and heart on-a-chip for investigating the link between Rheumatoid arthritis and the increased risk of cardiovascular disease. However, despite these efforts to replace animal models a series of different approaches are needed to fully determine the anti-arthritic and tissue reparative properties of new therapeutics.

Continued review of the scientific literature will be undertaken on a regular basis in order to identify any newly emerging technologies and models that could be potentially adopted in order to replace in vivo animal use.

Why were they not suitable?

The cell culture models that we currently have up and running can provide information currently from one or two cell types only and do not represent the complexity of the inflammatory response. It is not possible to conduct long term dosing studies or investigate the impact of disease on multiple organ systems with current in vitro models due to issues with cell survival in for example the small chambers required for culturing endothelial cells under flow.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals estimated for use is from our extensive experience in running these models over the past 5-10 years. We have been breeding the colonies specified in the PPL for over 10 years and understand the levels that we need to maintain our research output. This is combined with our wide use of in vitro cell culture methodologies, which helps us to reduce the number of animals required for our research outcomes.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will perform statistical power calculations to ensure that we use the minimum number of animals needed to obtain statistically significant results. This will avoid excessive use of animals. These calculations are based upon previous data following extensive use of the majority of protocols within the PPL. Generally using a significance level of 5%, a power of 80% and an effect size of 1.8, group sizes are typically 6-8 per group for acute

inflammation and arthritis protocols. In the absence of such data we will perform pilot studies to establish the number of animals required to test our hypothesis. In designing our experiments, we aim to ensure randomisation of animals to different treatment groups and we will try to ensure that people performing analyses are blinded to animal treatment groups as recommended in the ARRIVE guidelines. We will consult trained statisticians before using any new protocols and all staff performing animal experiments will attend appropriate training on key aspects of experimental design including the use of experimental design tools such as those at https://eda.nc3rs.org.uk.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Due to the complex nature of chronic inflammation and our chosen models we can compare processes on multiple organ systems within the same animal. This increases the power of our analyses and ensures that we get as much information as possible from each animal, which ultimately reduces their use. At the end of the experiment multiple organs and tissues will be harvested and analysed by PCR and flow cytometry and serum will be collected for identification and quantification of systemic mediators. We have projects ongoing on several different organ systems, hence we utilise as many organs from each mice as possible for different project/researchers. We have refined our ex vivo protocols extensively to ensure that as many parameters as feasible are assessed in each experiment without compromising the cumulative severity of individual animals.

We will continually seek to optimise breeding strategies to obtain sufficient numbers of GA and appropriate control animals in a timely fashion. In this we will follow guidelines from https://nc3rs.org.uk/generation-and-breeding-genetically-altered-mice and information such as https://research.uci.edu/forms/docs/iacuc/JAX-breeding-strategies.pdf as well as taking advice from our colleagues, our NACWOs and our NVS. Efficient colony management will ensure that only colonies that are actively being used are mated and producing animals. For some acute inflammation studies we will use inbred strains to reduce variability thus giving a greater statistical power from small group sizes.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice represent the best species to be used in these studies as all models and protocols proposed in this application have been extensively validated and optimised by the wider research community. This is important because it will reduce the number of animals required and the need to set up and optimise experimental protocols. Experiments in mice will be utilised to obtain further mechanistic and multi- system responses such that they complement our human studies. We have extensive experience with the murine models

within the PPL as well as with the use of transgenic animals.

Where we need to validate our findings in a second species we will use rat models due to the tools and models available when it is necessary to validate and extend our findings.

Why can't you use animals that are less sentient?

An important aspect of our research goal is to find new therapeutics that are able to reduce pain responses in patients with arthritis. Pain is a primary disabling feature of arthritis and the main symptom that brings patients to the doctors office. An ideal therapeutic drug would protect joint tissues from destruction, initate repair mechanisms and also provide rapid symptom relief. In order to test this we need to perform weight bearing tests on sentient and conscious animals. Although other animals such as zebrafish offer valuable insights into developmental biology and osteoarthritis genetics, their simpler joint structures and different biomechanics make them unsuitable for our current research goals. Mouse models of arthritis are well-established and widely used in research, making them reliable for studying the disease and testing treatments. There are extensive tools and resources for mouse research, supporting high-quality studies and speeding up the development of new therapies, while reducing the number of animals needed.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animal welfare is a key consideration in all of our protocols and we will be guided by our NACWOs and NVS in always ensuring that we are using best practice and the most refined techniques. All staff involved in animal experiments will review the literature on animal welfare provided by the local AWERB. Taking this into consideration as well as the aims of the PPL, which is to elucidate fundamental mechanisms at play in chronic inflammatory pathologies we will use animal models that best recapitulate the disease processes in humans. This is vital as it ensures any findings are relevant to the human condition.

Where possible use analgesics to reduce pain and suffering. In all surgical models pan-anaesthesia will be used. We will utilise disease severity scores to closely monitor animal welfare with clearly defined humane end-points. These scoring systems take into account animal condition and behaviour as well as clinical severity to enable monitoring of the whole animal. These will be coupled with measures taken to reduce suffering wherever possible (e.g. through provision of soft bedding and gel food). Along with the guidance indicated in our scoring systems we will always seek advice from the NVS/NACWO if animals reach early end points. Where studies in GA mice are anticipated to impact on disease severity we will take steps to reduce severity by using a lower dose of serum to induce arthritis or injecting a lower concentration of an inflammogen in a peritonitis model.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

In order to minimise distress, we will use a proactive welfare management approach that will follow guidelines set out in the publication 'Applying refinement to the use of mice and rats in rheumatoid arthritis research' by Hawkins and colleagues (2015).

This may include the provision of soft litter to reduce pain upon movement, the use of non-

tangling nesting material, housing with appropriate cage mates to reduce stress and enable thermoregulation, providing refuge as well as easy access to food and water.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We stay up to date with NC3Rs monthly newsletter and attend workshops; our PhD students recently attended the study design workshop.

36. The ability of biomaterials to form ectopic bone

Project duration

3 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

sub-cutaneous, biomaterial, bone regeneration, scaffold geometry, cell infiltration

Animal types	Life stages
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To screen the ability of a range of non-toxic biomaterials (as particles or scaffolds) to encourage tissue and bone formation through designed, instructive material properties (e.g. shape and mechanical properties). This will be done by placing the biomaterials in pouches under the skin (sub-cutaneously) of an adult rat. This will allow us to compare the amount of cell infiltration, new bone and blood vessel formation, that each type of material causes over a set period of time, and then select the best performing material to progress to a small animal bone defect model.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Large skeletal defects resulting from trauma, tumour resection, and disease remains a largely unresolved clinical problem, requiring a bone tissue engineering solution.

Therefore, there is an unmet clinical need to develop new therapeutic approaches for bone regeneration and blood vessel formation in bone defects. It is hoped that by screening a range of non-toxic and biocompatible materials we will be able to select one capable of supporting bone growth, cell infiltration, and the formation of blood vessels within a defect site. This benefits humans by potentially reducing the number of surgeries and infections associated with bone grafting and provide a material which encourages better quality bone regeneration within a defect site.

What outputs do you think you will see at the end of this project?

Data will be used to create publications which will be submitted to high quality reviewed journals. In addition, the data will be presented at international conferences. Best-performing materials will be progressed towards the clinic through pursuit of funding to create products based on them.

Who or what will benefit from these outputs, and how?

The data derived from this project will be used by scientific researchers, industrial partners, clinicians, and veterinarians.

Short term benefits (1 - 3 years) - the screening of products to allow set up and completion of the small animal bone defect study. Intellectual property protection of best performing materials.

Medium term benefits (3 - 5 years) – New knowledge, information, and data on biomaterials beneficial for this application shared through peer-reviewed journals and international conferences to benefit wider scientific community and facilitate additional funding to support the progression of this research.

Long term benefits (5 - 10 years) – Commercialisation and clinical translation of discovered beneficial biomaterials to address unmet clinical needs. Realised through large animal bone defect study building on previous data, successful regulatory approval of the material production (e.g. manufacturing, packaging, and storage), identification of suitable industrial partners.

How will you look to maximise the outputs of this work?

We will be collaborating with different research groups and any industrial partners to develop our technology further. In addition, we will be publishing the results of this study as well as presenting our findings in symposia and conferences.

Species and numbers of animals expected to be used

- Rats: 40

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The subcutaneous rat pouch model has been published many times and is the easiest surgically, with the least effect on health of all experimental models of ectopic bone formation. It is also the least invasive compared to the kidney capsule and muscle implantation models. Adult rats e.g. Wistar and Sprague Dawley are a good choice as they have a lot of loose skin which allow several implants to be accommodated. This allows many products to be screened for usefulness before commencing a bone defect study. There are no naturally occurring bone-forming stem cells under the skin; therefore, newly formed bone can be confidently attributed to the properties of the implant (a desirable characteristic). Therefore, it is a true test of the implant material, and we are able to test the ability of the designed scaffold shape, coating, or cells alone in their ability to form new bone. We are also anticipating differences in the quality and quantity of the new blood vessels and tissue that may grow into the implants.

Typically, what will be done to an animal used in your project?

Up to six materials will be placed in pouches under the skin (subcutaneously) of an adult rat. This will allow us to compare the amount of tissue and bone formation, and new blood vessels that each type of material causes over a set period of time and select the best performing material to use in a future bone defect model. 6 implants per group was selected and data generated will be analysed using a suitable statistical package and statistical tests. All experiments will be conducted and recorded in a manner that will allow high quality publication.

We have selected adult rats for this study as they are commonly used for subcutaneous implantation due to their relatively large size and loose skin capable of accommodating moderately sized implants without much discomfort to the rat. Adult rats of at least 250g will be group housed and acclimatised for at least 1 week before surgery therefore allowing their health status to be assessed.

All surgery will be carried out in a sterile fashion in dedicated facilities with experienced staff. Rats will undergo one surgery and will receive up to 6 sterile implants into 6 subcutaneous pouches on their back, avoiding the spine (see Figure 1). Pain relief and anaesthesia will be provided before an incision is made. There is a small risk of the incisions opening; therefore, we will include the option to re-close an inadvertent opening of each incision once, and anaesthetise the rat a maximum of 2 times after the initial surgery, The NVS will be consulted to assess whether a non-surgical closure e.g. skin adhesive or clips may be used.

Animals will recover fully in a heated and secure environment. Once fully recovered from anaesthesia they will be returned to group housing with behavioural enrichment unless we find their cage mates are damaging their incisions. Clinical scoring, including the rat grimace scale, will be used for the first 3 days post-operatively. Local numbing cream may be applied to wounds at any time in the study as advised by the NVS. Non-steroidal anti-inflammatories will be used, and antibiotics will not routinely be given unless advised by the NVS.

Blood samples, e.g. taken from the tail vein or via cardiac puncture, may also be taken at several time points, for example 1, 4, 8 and 12 weeks after surgery to look for a response to the implant. At this time rats will experience very brief discomfort from a small needle

inserted in the tail vein. The cardiac puncture technique for blood sampling will only be carried out on terminally anesthetised rats.

Our scientific endpoint is set at a maximum of 12 weeks, as without sacrificing the animal, we are unable to assess the bone and tissue growth and blood vessel formation into the implanted material.

What are the expected impacts and/or adverse effects for the animals during your project?

The creation of sub-cutaneous pouches and the delivery of biomaterials in rats is a minor procedure and the animals are expected to make a full and rapid recovery. They will receive analgesia and will be monitored closely for three days after surgery.

From previous studies carried out we do not expect the animals to show signs of pain and distress for a significant period of time, but if they are not showing signs of improvement, they will be killed to prevent any suffering. A scoring system will be used to monitor the animal's wellbeing after surgery. Delivery of any material to a subcutaneous pouch will be closely monitored to ensure that there is not significant inflammation, e.g. swelling and/or seroma formation. If at any point throughout the study an animal shows signs of becoming unwell the NVS will be contacted for advice and clinical scoring will begin.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

During this protocol 100% of the animals will suffer moderate levels of severity as a result of the surgical procedures conducted under general anaesthesia.

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Cell based laboratory (in vitro) work was initially carried out to ensure that the implants/scaffolds were not toxic to cells, but in vitro assays do not allow us to assess the physiological response to an implant and whether it will be capable of stimulating new bone formation and assessing the rate of cellularisation. For this reason we need to carry out this work in a living animal.

Which non-animal alternatives did you consider for use in this project?

In vitro testing was carried out to assess the biocompatibility and cytotoxicity of the material, but it is not possible to recreate the in vivo environment.

Why were they not suitable?

In vitro assays may indicate what the final outcome may be regarding cytotoxicity, but in vivo studies are required to test the item in a complex whole body system. In addition, the duration we require to test the materials for are not suitable for in vitro trials.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We will always seek to use the minimum number of animals necessary to achieve the objectives of the project whilst maintaining statistical validity of results. We have based the numbers of rats required on previous studies we have carried out, and on the published literature for this model that shows that sample size ranges between 4 and 6. We have therefore selected a minimum of 6 implants for each experimental and control group.

The positive control will be a material that is already proven and in clinical use, for example a porous ceramic or polyethylene material provided as cubes of a similar size to the test material. The negative control will be an inert, non-porous material that is the same size as the test material.

A pilot study will be carried out on 2 animals to ensure that placing 6 incisions and 6 implants does not cause harm and extend the recovery of the rats. If 6 implants and 6 incisions are too many, we will initially reduce to 4 implants and 4 incisions, and then further to 2 implants and 2 incisions if necessary. The data from the pilot study will be included in the study and is not in addition. Findings regarding the tolerance of up to 6 implants will be incorporated in publications to reduce the need for repetition by the wider scientific community in future, disparate studies.

In our research we will randomise our experiments to avoid bias, for example we randomise which animals get which treatment i.e. biomaterial or control and in what order they undergo procedures. We will analyse our data in a blinded fashion and use coding systems to anonymise data, thereby reducing unconscious bias. In some instances, we may ask our collaborators to randomly allocate animals to experimental groups and to retain the information as to which group and animal belongs until final results are collected. We believe that these methods contribute to the robustness of our data interpretation. We are committed to working to the PREPARE guidelines.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The NC3R Experimental Design Assistant was used to create a study plan. This allowed us to critique the plan and run a power calculation and assess the sample size required for this study.

We will also maximise the data obtained from each rat e.g. micro-CT data, histological and immunohistochemistry data.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

At the end of the study any tissue not required by our group may be harvested by other groups for further research.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The rat is commonly used for subcutaneous implantation as they are relatively large and have loose skin to accommodate a moderately sized implant without much discomfort to the rat. To reduce distress, rats will be group housed and acclimatised for at least 1 week before surgery. Pain and suffering will be minimised by carrying out all surgery aseptically in dedicated facilities with experienced staff. All animals will receive pain relief during and after surgery and antibiotics will be given if required. Once fully recovered from the anaesthesia they will be returned to group housing with behavioural enrichment. From previous studies carried out by our group at this facility we do not expect the animals to show signs of pain and distress for a significant period of time, but if they are not showing signs of improvement, they will be killed to prevent any suffering. A scoring system will be used to monitor the animal's wellbeing after surgery.

Why can't you use animals that are less sentient?

The most physiologically suitable vertebrates are mammals, and of these, adult rats above 250g are required to allow a big enough sub-cutaneous space to place the implants. It is expected to take several weeks to encourage cell infiltration and possible bone formation. Therefore, an animal that is terminally anaesthetised would not be suitable.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Assuming that the pilot study is successful, we will be able to place 6 scaffolds into subcutaneous pouches per rat and therefore reduce the number of animals needed to

complete the study. All surgery will be carried out aseptically in dedicated facilities, according to the LASA guiding principles for preparing for and undertaking aseptic surgery.

An animal will only ever undergo two additional anaesthesias to re-close incisions that inadvertently open, with each incision being closed only once and under NVS advice possibly using tissue adhesive or clips to avoid the additional anaesthetic event. All materials to be implanted into the rat will be sterile. Analgesia will be provided before an incision is made and after surgery as advised by the NVS, and local analgesic cream may be applied to wounds at any time in the study. Antibiotics will not routinely be given unless advised by the NVS.

Animals will recover fully in a heated and secure environment before being returned to suitable housing. The rats will be group housed with behavioural enrichment unless we find their cage mates are damaging their incisions. Clinical scoring including the rat grimace scale, will be used for the first 3 days post-operatively. Delivery of any material to a subcutaneous pouch will be closely monitored to ensure that there is not significant inflammation. If at any point throughout the study an animal shows signs of becoming unwell the NVS will be contacted for advice and clinical scoring will begin.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The project will be carried out under the following guidelines- PREPARE guidelines BVA/FRAME/RSPCA/UFAW Refining Procedures for the Administration of Substances Working Group report

LASA guiding principles for preparing for and undertaking aseptic surgery

NC3Rs guidance for *in vivo* techniques

All findings will be reported according to the NC3Rs ARRIVE Guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

During the study we will be in contact with the NACWO and the NVS in addition to monitoring any updates from the NC3Rs website.

37. Understanding the migratory patterns and functions of immune cells during inflammatory challenges

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

White blood cell (leukocyte), Neutrophil, Macrophage, Inflammation, Cell migration

Animal types	Life stages
Zebra fish (Danio rerio)	adult, embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Our overall aim is to understand the movement patterns of white blood cells (leukocytes) in vivo after encountering infection and how these help them eliminate microbes. In particular we aim to understand the processes and mechanisms that first escalate and subsequently resolve leukocyte accumulation at infected or injured lesions and how these migratory patterns may change from one infection to the next across an organism's life.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Every time we experience injury or infection, our body launches an inflammatory response in an effort to defend breached tissue from invading microbes. This is achieved through the recruitment of inflammatory cells, such as neutrophils and macrophages, which kill pathogens and repair tissue.

Despite the uncomfortable symptoms of inflammation, this process is so important to our survival that it has remained almost unchanged across vertebrates, from fish to humans.

However, the long life span of humans in the industrialised world has brought about a major pathological angle to inflammation: Age-associated diseases, such as cancer, neurodegeneration (e.g. Alzheimer's) and autoimmunity, lead to constant, low-level tissue damage, which can be detected by inflammatory cells and create a vicious circle of immune reactions that do not resolve. For this reason, targeting dysfunctional inflammation of this kind represents one of the biggest pharmaceutical investments of our time.

A major limitation in this effort is our lack of understanding of how inflammatory responses naturally unfold in vivo. For example, although we have some understanding of how these responses are initiated through the recruitment of white blood cells to damaged tissues, we lack knowledge on the migration behaviours thereafter and how the overall reaction resolves. This makes it difficult to predict and manipulate the accumulation of inflammatory cells in a specific manner in disease. We also lack information on how the fates and migration patterns of inflammatory cells in the body after infection influence subsequent challenges. It is essential to understand this, as real-life inflammatory reactions occur in the background of prior microbial encounters and it is becoming increasingly clear that an individual’s infection history can influence subsequent inflammation.

What outputs do you think you will see at the end of this project?

Our main output will be new fundamental knowledge of immune cell functions in the form of peer- reviewed publications, presentations in conferences and scientific protocols. New collaborations and projects building on this new knowledge will be another output.

Who or what will benefit from these outputs, and how?

Short-term: The work will in the first instance have an impact in the field of immunology and cell biology.

Long-term: The long-term impact of the work is in the area of immunotherapy (whereby immune cells are taken out of the body, modified to achieve a certain function better, such as tumour elimination, and re-infused into a patient) and vaccination. For example, identification of cell migration mechanisms of immune cells have relevance in cancer immunotherapy, as such mechanisms can be exploited to enhance migration of the desirable immune cells to tumours. Our studies on immune cell training can generate new knowledge that can inform the design of future vaccines against infectious agents or tumours.

How will you look to maximise the outputs of this work?

We will publish our findings and related methodologies in peer-reviewed journals and conferences. This will often include unsuccessful approaches that may help others orient or reduce their experiments. We will establish collaborations to help translate our findings to other mammalian or human studies, which may pave the way for long-term application in clinical settings.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 213,300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The project aims to unravel the journeys of immune cells in the whole body of an animal during the whole course of an infection, therefore requires animal usage. We are using the zebrafish larva, which is the most refined choice for these studies, as opposed to mammalian models. As a vertebrate, zebrafish larvae have immune cell mechanisms that are highly conserved; in particular the more ancestral first line of defence, referred to as 'innate immune system', that includes cells like neutrophils and macrophages. In contrast to mammalian models, the zebrafish larva is transparent allowing us to non-invasively image the behaviour of immune cells during infection. To visualise immune cell behaviour and interrogate related mechanisms, we need to introduce genes reporting on their function or genetic mutations. For a relatively small number of experiments we are using adult zebrafish that will be subjected to agents hypothesised to improve/train innate immune cells (neutrophils/macrophages). This is necessary in order to obtain sufficient numbers of immune cells for transplantation experiments (conducted in larval recipients) or for the study of these cells outside the animal (in vitro). These experiments are important for understanding the mechanisms that alter the properties of these cells such that they fight pathogens better.

Typically, what will be done to an animal used in your project?

The adult zebrafish will be housed in a dedicated aquarium within the department, run by trained staff. We will generate genetically altered zebrafish by introducing modified genetic material in fertilised eggs and growing these embryos to adulthood.

In order to know which fish contain genetic alterations, we sometimes need to carry out genetic analysis via e.g. cutting a small portion of the fish’s tail fin under general anaesthetic and analyse the genetic code inside this tissue. The fish is then kept in a separate tank with fresh water and the fin then regrows relatively quickly (within approximately 2 weeks). Once the genotype is known, the fish will be paired with at least one other fish in tanks of appropriate size.

Where appropriate, other methods of genotyping may be used, such as swabbing the surface layer of the skin. Adult fish will be maintained until a maximum of 30 months of age. During this time, adult fish will be bred in specialised breeding tanks to enable the production of genetically altered zebrafish embryos. We very occasionally need to anaesthetise fish for the collection of eggs and sperm. For a relatively small number of experiments we are using adult zebrafish, that will undergo non-harmful immunogenic challenge, similar to vaccination. This is because adult zebrafish serve as a better system for isolating immune cells and performing in vitro studies or transplantation experiments than larvae (the number of immune cells in one adult fish is several orders of magnitude higher than in larvae).

Microscopy imaging, tissue injury and infection experiments will be done in larval stages, at which the animals don't have fully developed cognitive abilities and stress responses (as an indication, most studies using zebrafish as a model for mental health disorders focus on adult zebrafish stages). In some experiments, fish larvae will be subject to brief, mild heat treatment a few degrees above their normal temperature. This will be done under general anaesthesia from which the larvae will fully recover. The effects from heat treatment are not expected to cause any adverse effects that are more than mild and transient. For a certain number of days after onset of independent feeding, larvae can uptake nutrients from the yolk sac. Therefore, to minimise detrimental accumulation of biological waste from the larvae in those stages, we will withhold food administration. In a small number of experiments in larval stages, cancer cells will be injected to monitor relevant immune responses. The effects of the transplantation will be closely monitored by following the cells microscopically. The dose of cancer cells to be transplanted will be optimised to reduce the clinical signs to the minimal.

For a relatively small number of experiments, we will perform non-harmful administration of agents in adult zebrafish, analogous to vaccination, most often through superficial administration in the skin or muscle. This will allow us to isolate sufficient numbers of immune cells to study their properties and behaviours outside the animal. For a small number of adult fish we will perform microscopy imaging during temporary anaesthesia.

At the end of the protocols, fish will be humanely killed or supplied to other project licences or recognised establishments with the authority to breed and maintain genetically altered zebrafish of this type.

What are the expected impacts and/or adverse effects for the animals during your project?

We do not usually expect there to be adverse effects to adults caused by the genetic alterations that we introduce, as most genetic mutations we introduce will be specific to immune cell mechanisms and we control the level of microbials in our fish facility.

However, it is possible that some level of immune dysfunction or unspecific developmental effects happen, leading to alterations in morphologies of the fish (e.g. thinner bodies) or higher susceptibility to infection.

We do not expect there to be any adverse effects from breeding the zebrafish. It is unlikely but not impossible that fish might develop an infection following removal of a small part of the tail fin, in which case we will humanely kill the fish. For genotyping and sperm/egg collection it is possible that fish may not recover from anaesthesia, but this is very unusual (less than 1%).
In regards to imaging and infection experiments in larval stages, we expect clinical signs of disease but we will titrate the dose of microbial pathogens to minimise clinical symptoms.

In the case of immunogenic challenge in adult zebrafish, we will titrate the dose of immunogenic agent so as to have minimal and resolvable clinical symptoms. As with genotyping, these injections carry a small risk of non-recovery from anaesthesia or infection (<1%).

In regards to the cancer cell transplantation experiments in larval stages, we expect some clinical signs of disease (small skin lesions) but they will be closely monitored and kept to

a minimum.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild: 99%
Moderate: 1%

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The central aim of the project is to track immune cells as they fight infections in the living body in physiological conditions. These include migration across different tissues in the body and interactions with all the different cells that shape their behaviour in vivo. Therefore, these experiments can only be performed in an animal.

Which non-animal alternatives did you consider for use in this project?

In collaboration with other labs, we are also complementing our work with in vitro human models and computational modelling approaches. Human in vitro work corresponds to about 5% of animal replacement and in silico, computational models correspond to about another 10%.

Why were they not suitable?

Although it is possible to study some properties of immune cells in a culture dish, the knowledge on how these cells get to acquire these properties during an immune response has to be obtained from the live animal, where we can follow cell interactions with microbes in the whole body. The computational modelling can provide useful predictions and hypotheses, but their relevance and importance has to ultimately be tested experimentally in the animal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Breeding:
Animal usages was based on requiring approximately 100 fish per new generation of fish for each genetically modified line. We will make a new generation for each line every year (5 generations per line). In addition, when generating new genetically modified lines, the F0 embryos need to be genotypically screened. Therefore, approximately 200 additional fish may be required per new line at the F0 stage to find appropriate founders to generate the F1 generation. Based on raising 20 established lines and generating 15 new genetically modified lines over the course of the 5 years, this makes approximately 13,000 adult fish. Up to 300 of these will be used for gamete collection.

Experiments in larvae:
Up to 400 eggs per experiment may be used. 100 experiments per year (approximately every two weeks on average across average of four users). This adds up to 40000 larvae. Multiplying this by 5 years is 200000.

Experiments in adults:
Up to 20 animals per experiment (10 per condition). 12 experiments per year, 60 over 5 years. This makes a total of 1200. However, these animals were already accounted in protocol 2 or 3 and therefore not included in the total number of animal used.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will carefully design our experiments so that we use appropriate numbers of embryos for each experiment. Where necessary and possible, we will carry out pilot studies to determine the number of embryos required to achieve robust statistical analysis. If we require assistance in our experimental design, we will consult with a statistical expert. We will ensure that our publications conform to the ARRIVE guidelines: https://www.nc3rs.org.uk/arrive-guidelines.

To make our experiments robust, we will control for variability in the following ways:
We will reduce environmental variability by carefully housing breeding adult fish in the dedicated zebrafish facility and by keeping genetic background constant within each genetically modified line of fish.

We will assess normal levels of variability within experiments via pilot experiments, allowing us to select appropriate statistical methods and number of embryos.
We will include randomisation, like using different clutches of fish, to ensure robustness of data across different biological samples.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We are using several methods to reduce the numbers of adult animals used:
- we will share relevant fish stocks with other users within the facility.
- we will try to limit repeated breeding to once per week to optimise breeding performance.

- we will make the most use of individual fish breeding lifetime and keep fish as long as they show good breeding and wellbeing with a maximum of 30 months.
- we will freeze sperm from genetically altered lines of zebrafish for longer-term storage.
- we will use the same breeders for tissue extraction experiments
- We will become part of the internal tissue sharing resource to facilitate this communication and optimise the number of animals used in our research

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use zebrafish because the immune cell mechanisms are highly conserved across vertebrate species and the transparency of larvae allow us to follow their behaviour during inflammatory challenge.

A significant part of experiments is performed at an early stage of life development (zebrafish embryos younger than 5 days old) which do not require protection in law.

A further part is performed at later larval stages that are less developed that full adult stage (less than 30 days old). Thus for the majority of the work, we are not using fully developed animals with fully developed responses to pain, suffering, distress and harm. In these stages, will use infection models that will induce some clinical disease symptoms, but we will aim to reduce any potential suffering of these embryos by titrating doses of agents used and promptly killing them using a humane, approved method at the end of the experiments. A minimal number of animals will undergo mild immunogenic challenge to study how we can improve the properties of immune cells by such agents. Importantly, the scientific aim here is to improve immunity with minimal distress to the animal.

Why can't you use animals that are less sentient?

A vertebrate species is needed to study the cells of interest (neutrophils and macrophages) as these cell types are not present in lower life forms (e.g. fruit flies).

We intend to perform the majority of manipulations on larvae younger than 5 days post fertilisation (dpf) and only in a subset of experiments we will keep larvae up to 14 dpf. At these early developmental stages, the zebrafish larva is sufficiently complex for the purpose of our study yet not as fully capable of cognitive interactions as an adult, therefore the protocol is mild. The reason for keeping larvae longer than 5 dpf is because after infection the course of disease outcome needs to be observed over several days to find out how the response resolves and how immune cells get trained for subsequent challenges. Our choice of model is therefore the most refined possible for answering our questions on leukocyte migration mechanisms in vivo.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We don’t envisage any suffering in the vast majority of licenced animals beyond mild procedures (i.e. breeding of genetically modified fish, infection challenge in larval stages and administration of immunogenic agents in adult stages). Moderate effects (for example, increased susceptibility to infection) might occasionally arise due to the genetic alteration of the fish or in pilot titration immuno- challenge experiments in adult fish. If that occurs, the animals will be promptly killed using a humane method and subsequent breeding practices or dose of agents will be adjusted. Monitoring and scoring sheets will be used to keep track of the wellbeing of the fish undergoing procedures. We will only use larvae up to 14 dpf for imaging or infection/injury challenge, and these stages are less sentient than adult stages. We will aim to reduce any potential suffering of these embryos by promptly killing them using a humane, approved method at the end of the experiments. Aside from using mostly larval stages for the infection and injury experiments, we will aim to use the mildest possible immune challenge in the few adult fish that will be used for immune-training/vaccination experiments. For example, intradermal/subcutaneous/intramuscular injection route and non-pathogenic agents will be preferred over more invasive routes and pathogenic microbes.

Adult fish will be housed in a dedicated centralised zebrafish facility, where they will be looked after by full time staff, who will ensure their welfare. Numbers of fish per tank, water quality and food quality and quantity will be optimised and carefully controlled. Our breeding protocols are regularly considered in terms of refinement through our zebrafish users meeting. In this meeting, all users of the shared zebrafish facility report issues of husbandry (general trends in survival, growth or breeding rates) and we discuss and implement better ways of breeding the fish. For example, we have recently refined our fin clipping to remove only a very small region of the fin, that alleviates the need to provide analgesia. Changes relating to husbandry practices are made in a consensus manner rather than in a project licence-specific manner and in consultation with the local veterinary advisor and animal welfare experts.

A key part of breeding GM zebrafish, is the need to screen for the genetic modification. Phenotypic screening before 5 dpf will be the preferred method (as a large proportion of the fish we will generate will have fluorescent reporters) and genetic screening of biopsies from adult fish will be used at a relatively low frequency.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

There are several resources to inform us about the current research on refinement of procedures (e.g. https://norecopa.no/species/fish/, https://nc3rs.org.uk/3rs-resources/zebrafish-welfare, https://www.lasa.co.uk/current_publications/, NORECOPA’s PREPARE guidelines: PREPARE (norecopa.no) and the 3Rs Zebrafish welfare resource: Zebrafish welfare | NC3Rs). These will be taken into account when deciding on the most appropriate method for procedures.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Advances in 3R tools are internally circulated. We can also access advances via the NC3Rs (https://nc3rs.org.uk/resource-hubs) and Norecopa website pages (https://norecopa.no/databases- guidelines). If scientifically appropriate advances in 3Rs arise in the course of the project, we will seek advice from the named veterinary surgeon, name information officer and named animal care and welfare officer about whether and how to implement them.

38. Breeding and maintenance of wild type and genetically modified mice for the investigation of G protein coupled receptor function in normal physiology and disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - (ii) Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

GPCR, Breeding, Maintenance, Genetically Modified

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant
Rats	embryo, neonate, juvenile, pregnant, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project licence will enable the breeding and maintenance of wild type and genetically modified mice as well as the harvesting of tissues and transfer of mice to other project licences. This will allow for the investigation of the biology of signalling pathways and an understanding of whether receptor molecules can be targeted with drugs as a novel therapeutic strategy.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could

be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cells in our body communicate with each other by releasing chemical messengers from one cell that then bind to and activate receptor proteins on other cells. One of the largest family of receptor proteins are the G protein coupled receptor (GPCR) family which include well known receptors such as those that bind to adrenaline, serotonin and dopamine. Because these receptors are on the outside of cells and because they are involved in many physiological and disease functions the pharmacological industry have been targeting these receptors with new drugs. In fact, GPCRs have been the most successful drug targets known to man. Despite this the full potential of the GPCR family has yet to be realised.

The reason for this is that we lack fundamental understanding of the biology of many of these receptors and have little knowledge regarding how to target them in disease. We lack effective drugs for the treatment of many of the world's most devastating diseases. This is exemplified by dementia where the treatments for relieving symptoms, such as memory loss, have limited efficacy whilst there are no drugs that stop or slow the disease. Currently, GPCRs offer a huge potential as targets for drugs for the treatment of many human and animal diseases including dementia. However in order to realise this promise we require a better understanding of fundamental aspects of GPCR biology, how receptors operate in disease and how best to design drugs with the correct pharmacological properties to treat disease whilst minimising side effects. Hence the breeding and maintenance of genetically modified mice described here will allow for these key studies to be conducted.

What outputs do you think you will see at the end of this project?

This licence is for breeding and maintenance. The outcomes described below will be from other projects that will receive animals from this licence .

1. In the neuroscience project we aim to establish the role of GPCRs in the regulation of learning and memory, anxiety-like behaviours and locomotion. We also expect to determine the impact of GPCRs particularly the muscarinic receptor family in the symptoms and progression of neurodegenerative disease and schizophrenia and addiction behaviour.

2. In the lung respiratory project we expect to determine the role of GPCRs in the regulation of lung function and if targeting GPCRs can change the course and symptoms of inflammatory airway disease

3. In the gut physiology experiments we expect to determine the role of GPCRs gut physiology and visceral pain.

4. In the opioid use disorder experiments, we expect to determine the role of GPCRs in drug seeking and reward pathways.

These discoveries will be disseminated in the following ways;

1. Peer review literature

2. Scientific meetings in the form of talks and poster presentations

3. To the general public in the form of press releases, public seminars and social media

We also expect these discoveries to result in further grant applications and both charitable and government grants.

Who or what will benefit from these outputs, and how?

Academic Community - will benefit from a understanding of fundamental biology of GPCRs and the understanding of the best ways to target GPCRs to regulate pathophysiological responses.

Pharmaceutical/drug discovery community – will benefit from the validation of new GPCR targets in human disease and an appreciation of the pharmacological principles that can be applied to drug design.

General public – will benefit from the prospect that new methods will be developed to apply to drug discovery against some of the currently most intractable diseases including Alzheimer's disease and inflammatory gut disease.

How will you look to maximise the outputs of this work?

In terms of publications in the scientific literature and presenting in research meetings we are very experienced in these areas with strong relationships with editors of the top journals including Science, Cell and Nature as well as being well connected with organisers of major research meetings. Hence we anticipate that we will continue to have strong outputs through these routes. We have also been developing our outputs via social media with both institutional and personal social media outlets being developed including Twitter and Facebook. Finally we are improving of public outreach with visits to local prisons, schools and presenting to Scottish Members of Parliament.

Species and numbers of animals expected to be used

- Mice: 45,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using mice for the following reasons;

1. Mice are a species where it is possible to change genes in a process often described as genetic engineering. In this way we are able to change specific genes that encode for proteins such as the receptors that are being studied in this project. Hence by genetically engineering mice we can either delete receptor genes or change them so

the receptor can be activated by synthetic drugs. This will allow us to probe the function the receptors. Such approaches are not currently possible with other species.

2. Using embryos and neonates are the optimum life stages to generate primary neuronal cultures using schedule 1 methods.

Typically, what will be done to an animal used in your project?

Typically the animals will be mated, litters weaned and offspring used in subsequent mating. Alternatively, offspring may be used for tissue or transferred to other project licenses. Tissue samples will be taken regularly for genotyping.

What are the expected impacts and/or adverse effects for the animals during your project?

We are expecting no adverse effects of animals bred and maintained in this project.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

the expected severity limits under this licence are 80% subthreshold and 15% mild due to requirement for repeated genotype sampling. The remaining 5% are categorised as non-recovery due to perfusion.

What will happen to animals used in this project?

- Used in other projects
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There are no available cell culture models to investigate the three primary areas of this project namely; (i) neurological disease, (ii) gut inflammatory disease and (iii) lung inflammatory disease. Although we are using human tissue samples as much as possible we require animal model of these diseases in the background of genetic models expressing mutant forms of our target receptors to investigate the role of GPCRs in the context of normal physiology and disease.

We are employing replacement strategies where possible such as those below;

1. By conducting biochemical experiments such as large scale study of proteins (proteomic) and the study of all RNA molecules in tissues (transcriptomics) we are

looking for early markers of disease that can give reliable indications of drug efficacy thereby reducing the number of animals and the time they are exposed to disease. This is particularly the case in the neurodegeneration studies but also in our inflammatory models.

2. We are using human tissue with increasing frequency to reduce the number of mice used.

Which non-animal alternatives did you consider for use in this project?

We are using human brain tissue (both normal and disease) obtained from registered human tissue banks for the neuroscience projects described here. We are also working closely with clinicians to obtain human lung tissue (both disease and normal) and human gut tissue. We are therefore making every effort to use human tissue to determine the role of GPCRs in normal physiology and disease as opposed to animal tissue.

Why were they not suitable?

Human tissue is preferable to mouse tissue however it is not possible to employ genetics to validate the receptor targets in human tissue. It is also not possible to trial drugs that target our receptors in humans - rather we can only test the response to our drug treatment in resected tissue or from postmortem samples. Hence we aim to combine the animal studies with human tissue studies to probe the function of GPCRs in human disease. The ultimate aim will be to subsequently develop drugs based on our findings to trial in human clinical studies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have ~25 mouse strains at any one time, on average maintained at 30 animals per strain per month. Which over a 5 year period equates to 25x30x12x5 = 45,000 mice.

These numbers are estimated based on prior experience of managing colonies to provide sufficient animals to conduct our intended experiments for the project licences we hold.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We are constantly making attempts to reduce the number of animals in the following ways;

1. We are using well described protocols which reduces the need for optimisation in pilot studies, consequently fewer animals are used.

2. We have established a strong and robust management system to oversee breeding

and maintenance to ensure the most efficient use of animals. This management system involves two dedicated animal technicians that maintain records and inspect the animals on a weekly basis.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have excellent management systems and data-bases in place to ensure efficient breeding and husbandry of the mice.

Where possible we also share tissue amongst users and importantly co-ordinate studies to most efficiently use mice. We also have a large tissue archive which is well indexed and stored.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

GPCR mutant mice

These will be used to directly determine the role of GPCRs in normal physiology and in disease. In particular we have developed mutant receptors that can not be activated by the natural ligands but instead be activated by drugs. In this way we can not only test the role of the target receptors in normal biology but importantly test the action of drugs that work by activating these receptors. Also we have developed GPCR mutant mice where the receptor is restricted in the number of signalling pathways that can be activated. Such receptor mutants can be used to determine the biochemical and signalling pathways used by receptors to mediate clinically important effects and distinguish these from pathways that lead to adverse responses.

Breeding and maintaining these animals will not lead to any pain, suffering or harm.

Neurodegeneration models

We will breed neurodegenerative models including prion over expressing and prion null mice. There is no suffering or harm associated with the breeding and maintenance of these strains. These strains will be used to determine the impact of GPCRs in the progression of disease and disease symptoms and whether targeting these receptors can modify disease.

Reporter mice for reactive oxygen species

There is no suffering or harm associated with the breeding and maintenance of these strains which will be used to monitor mitochondrial function during neurological disease particularly prion disease and whether targeting GPCRs can impact on mitochondrial activity.

Why can't you use animals that are less sentient?

Where possible we will use early life stages (e.g. mouse embryos and neonates) to generate neuronal cultures and terminally anaesthetised animals for histology (e.g. perfusion fixation). However the neuroscience projects require models that most closely resemble human physiology and be models that can be genetically manipulated in this case mice are the most appropriate. Also we wish to test the action of receptors and drugs that might lead to new drugs for human use. The receptors and receptor system need therefore to closely relate to humans and therefore mammalian systems such as mice are the most appropriate.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We are always looking for further refinement of procedures. For example we have been undertaking a study to determine biomarkers of neurodegeneration that can be used to establish early on in disease if targeting our receptors of interest impacts on disease before clinical signs appear. We have also been looking towards methods of evaluating gut inflammation using mild inflammatory models and assessing sensory neuronal activity using terminally anaesthetised animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Our primary published source of guidance on 3Rs is via the national centre for replacement, refinement and reduction in animal research (NC3Rs). This organisation publishes regularly on guidance for researchers. The European Medicines Agency also publish excellent practical guidance on 3Rs. We also pay particular attention to the peer reviewed scientific literature for further methods to refine our protocols.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have regular up-date sessions and training in new approaches run locally and nationally. We also keep abreast of the published literature and share good practice locally. Importantly, we also have expert collaborators that share good practice and we are always looking at new methods to improve our 3Rs.

39. Exploring the functional heterogeneity of skin cells in development, regeneration and disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Skin cells, Wound healing and fibrosis, Cancer, Skin disease, Therapy

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The project explores how different skin cell types influence each other during development, regeneration, environmental stress and disease, such as fibrosis and cancer. We particularly focus on dissecting the functional heterogeneity of skin fibroblasts and explore their therapeutic potential including gene editing approaches.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Skin fibroblasts play a central role during skin homeostasis, wound healing and diseases including chronic wounds, skin fibrosis and cancer with great clinical unmet needs. Understanding their functional heterogeneity and interaction with other skin cell types will

pave the way for novel fibroblast targeted therapeutic opportunities.

What outputs do you think you will see at the end of this project?

Output will include new knowledge, publications, omics datasets and mechanistic insights into vital biological processes of the skin during health and disease and new emerging gene therapy approaches for congenital skin diseases. These will support funding applications and preclinical packages for translational development which will be essential for patient benefit. Where opportunities arises we aim to create IP of new therapeutic applications.

Who or what will benefit from these outputs, and how?

In the short term the scientific community will benefit from the new knowledge and datasets generated during the project leading to new scientific advances in the skin field and beyond. Further our work will support multiple funding applications and public engagement activities.

In the mid/long term the project will enable training the next generation of scientists in key in vivo and transferable skills and strengthen the UK life science infrastructure.

Ultimately the project will enhance new therapeutic and diagnostic developments for diseases of the skin and potentially other organs leading to a direct patient benefit in the longer term.

How will you look to maximise the outputs of this work?

Our outputs will be disseminated at conference presentations, workshops, meetings with collaborators and in publications in high-impact journals and appropriate databases. Where possible we will also publish unsuccessful approaches and disseminate generated protocols.

Species and numbers of animals expected to be used

- Mice: Breeding: 5000 Experimental: 4200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Currently it is not possible to fully recapitulate the heterogeneity and complexity of organs systems in in vitro models to dissect developmental processes and disease pathologies or to test novel gene editing approaches in the skin and other tissues. Mice have been selected because they are a well-validated experimental model that mimics key aspects of human skin physiology and disease. This includes the complex cellular interplay between different skin cells in homeostasis, wound healing and disease such as fibrosis and cancer. Mice are well adapted to living and breeding under laboratory conditions allowing timely

and cost-effective disease model analysis which are highly relevant to human skin physiology and pathology. Mice can be genetically manipulated to inducibly deplete disease causing genes and allow to monitor disease trajectories.

Typically, what will be done to an animal used in your project?

1.Generating and maintaining genetically altered (GA) mouse models to study cell behaviour during skin development, wound healing and disease:

GA mice will have specific manipulations that label different skin cells or measure biological activity of signalling pathways or modulate expression of a protein. While 95% of GA mice will not develop a harmful phenotype during breeding, only in selected skin disease models GA may develop a transiently mild to moderate skin phenotype after birth. Tissue biopsy to determine genetic status (e.g., by ear punch) will be performed.

2.Dissecting the role of different skin cell populations in skin homeostasis, regeneration and disease:

Mice of different ages may receive labelling agents in order to identify specific cell subpopulations or modulate protein expressions by most appropriate route. To explore the skin response to environmental stress and regenerative potential, skin may be challenged by wounding, exposure to controlled UV irradiation or repeated application of a disease causing agent via appropriate route. It is very unlikely that more than one procedure will be carried out on the same mouse and selected challenges may require short term analgesia and anaesthesia (AB). All mice will be evaluated macroscopically by visual inspection. Some mice will receive a treatment after a skin challenge to modulate a pathological skin response and skin may be monitored at specific intervals with non- invasive imaging techniques requiring anaesthesia (AB). Blood may be taken at different treatment/disease stages. Various mouse tissues will be analyzed after Schedule 1 at the end of the procedure after up to 6 months.

3.Functional analysis of specific skin cell populations for skin regeneration and homeostasis

For functional analysis of an identified subpopulation, isolated cells will either be transplanted with a chamber or directly injected into the skin in immune compromised mice or mice with identical genes. Blood may be analyzed. In some occasions skin with transplanted cells may be challenged by either UV irradiation, application of substance or wounding. All mice will be evaluated macroscopically by visual inspection and in some occasions could be subjected to non-invasive imaging techniques requiring anaesthesia (AB) before being humanely killed for tissue analysis after up to 4 months.

4.Exploring fibroblast contribution to skin cancer development and progression:

To elucidate the contribution of different dermal fibroblasts or a gene of interest to tumour formation, reporter or gene specific transgenic mice will be crossed with a cell population-specific targeted line and treated with a gene inducing agent. Skin cancer will be induced by either repeated application of a topical carcinogen, induction of an oncogenic mutation or transplanting tumour cells on immune compromised mice and will be maintained for a maximum of 2-6 months depending on the tumour model. Some mice will be treated with an agent via one or more routes to monitor or modulate tumour progression and blood may

be analysed. Some non-invasive imaging and skin phenotype analysis procedures may require brief anaesthesia (AB). Mice may be injected subcutaneously or intraperitoneally with innocuous cell trackers before killing to assess cell proliferation and blood might be taken at various points.

5.Testing skin cell targeted gene therapy for genetic skin diseases

For genetic skin diseases such as Epidermolysis bullosa, gene therapy is the only possible cure. While new technological advances with gene editing tools such as CRISPR/CAS or base editor system show promising results in cell culture, the specific and efficient delivery to the skin in vivo remains a key challenge. To develop a skin cell specific delivery system of gene editing tools, we will test different treatment strategies in GA mice carrying disease causing genetic mutations and mimicking the human disease. All mice will be evaluated macroscopically by visual inspection and in some occasions could be subjected to in vivo imaging or blood sampling. Gene corrected skin may be challenged by wounding or tape stripping before being humanely killed for tissue analysis after up to 6 months.

What are the expected impacts and/or adverse effects for the animals during your project?

GA animals are not expected to develop a harmful phenotype; however, adverse effects may include transient weight loss (typically up to 15%), as reported by the dosing of some transgene activators, which recovers over time. Animals may show signs of general malaise, such as hunched postured, piloerection, inappetence, reduced mobility and reduced general activity. Typically, this is transient and animals improve over time.

A small number of mice may develop a harmful mild to moderate phenotype due to ablation of a specific cell or signalling molecule. Therefore, all mice will be monitored closely and any mice where a harmful phenotype becomes evident and likely to exceed moderate, will be terminated.

Applied skin challenges may result in a transient or permanent impairment of normal skin function. In an unlikely event of severe adverse effect any animal reaching one of the following humane endpoints will be humanely killed using a Schedule 1 method. These include when an animal show persistent deviation from normal health (>48 hours) such as piloerection, hunched posture, diarrhoea, dyspnoea or loses 15% of its body weight compared to age matched controls.

In the unlikely event (less than 1%, in our experience) of post-operative complications, mice will be killed unless, in the opinion of the NVS, such complications can be remedied promptly and successfully using no more than minor interventions.

Rarely, applied agents may cause skin inflammation, itchiness, flaking or erosion. In these cases veterinary advice will be sought on symptomatic treatment and if these conditions and/or erosions do not improve within 24 hours of treatment the mice will be subjected to schedule 1 killing.

GA animals treated with a skin carcinogen or transplanted with tumour cells will develop skin cancer that are no larger than 1 cm in any dimension and are few in number (less than an estimated equivalent of 10% of body weight in total). However any animal that experiences discomfort as a result of the site of a tumour (e.g. around the eyes, mouth,

anus, footpad), tumour burden or shows tumour ulcerations with necrotic areas resulting in long-term skin break down (non-responsive to healing >24 hours) or other impaired body conditions (e.g. persistent hypothermia, anaemia or significant abdominal distension) will be killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most GA breeding (95%) will be subthreshold/mild with only selected transgenic disease models leading to a transient moderate phenotype. All induced skin impairments will be within mild to moderate severity. As our key focus is on the early stages of tumour development, most GA animals treated with a topical skin carcinogen or transplanted tumour cells may develop a moderate phenotype. Specific monitoring, and care protocols to regularly assess body weight and clinical welfare parameters will be carried out (weekly to daily as tumour onset and development is timely expected). Humane endpoints (e.g., body weight loss up to 15%) will be applied to ensure no clinical signs will progress beyond moderate.

What will happen to animals used in this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The goal of this project is to understand the molecular mechanisms controlling skin cells during development, wound healing and disease. To study these complex processes and dissect the profound signalling crosstalk at different developmental, regeneration and disease stages cannot be undertaken without using laboratory animals. In particular the coordinated behaviours on the tissue scale of multiple cell types including cells of the blood vessels, immune system and connective tissue in skin, are not adequately mimicked in cell culture at this stage. Similarly, the development of new therapeutic approaches such as fibroblast subpopulation therapies or skin specific gene therapies require the use of disease models that fatefully recapitulate key features of the human diseases.

Which non-animal alternatives did you consider for use in this project?

Wherever possible we are incorporating cell culture models to replace or complement our in vivo work. However it is a well-recognized that freshly isolated mouse fibroblasts lose their specific function and become rapidly activated outside the body. Thus we are currently establishing culture conditions to preserve fibroblast identity and function. Preliminary data indicate that by culturing freshly isolated fibroblasts on culture dishes with

physiological stiffness, different types of fibroblasts can be expanded while maintaining their individual functions. In addition we have established 3D skin and decellularised dermis (DED) cell culture models which represent ideal tools to study skin cells outside the body in 3D environment. We regularly use them to screen new drug candidates or validate our in vivo findings in human cells where possible. However, these cultures have limited lifespan of approximately 4 weeks, co-culture of different populations is very challenging and thus only partially mimic normal tissue function. In addition the development of computational tissue models that recapitulate key aspects of skin biology that we have successfully used in the past will help to generate more targeted hypothesis and reduce the use of animals in the future.

Why were they not suitable?

Where possible we replace mice with studies of cells that are grown in culture in the laboratory and our experiments will be supported by computational tissue models. However, there are three situations in which cultured cells cannot be used: (1) when the properties of the tissue need to be studied for over 3 weeks, which is the limit for maintaining skin cells in culture; (2) when communication between multiple different cell types within the tissue, such as epidermal, nerve and blood cells, needs to be analysed; (3) when the development, progression and treatment of diseases such as cancer or fibrosis needs to be tested.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

All experiments have been designed with reference to the PREPARE guidelines (Norecopa) and the ARRIVE 2.0 reporting guidelines published by the NC3Rs. We will ensure that all animals are maintained on an applicable genetic background and housed under the same conditions with environmental enrichment provided. By using matching mouse strains, we will be minimizing variation between experimental groups and maximise reproducibility. Where applicable we will constrain groups to age, weight, and sex matched animals. This does not restrict groups to single sex animals but makes sure that equal composition of groups is maintained.

During the experimental design we will engage with a statistician to ensure that we have a clear strategy to generate and process the resulting data. At every stage we will challenge our datasets to screening raw data for obvious errors such as imputing/typographic errors and analysis of variance with comparable, existing datasets. All raw data will be recorded and archived appropriately to allow follow-up statistical studies to minimize animal usage.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To support our experimental design strategy, improve scientific efficiency and minimise animal use, we used the strategic planning for research programmes developed by FRAME, and the NC3R's Experimental Design Assistant. We further used the 3Rs databases ALTWEB and AWIC to search for alternatives to Animal Testing and improve animal care strategies.

For most of the quantitative experiments sample size will be supported by statistical analysis using the Experimental Design Assistant from NC3Rs to ensure that we use the minimum number of mice per group. Here we aim to achieve a minimum power of 0.8, assuming statistical significance of P<0.05. If necessary further specialist advice on experimental design and statistical analysis will be obtained from the statistical service unit at QMUL. Otherwise, we will use our previous experience (ours or from the literature) to select sample sizes.

In accordance with the ARRIVE 2.0 guidelines, randomisation and blinding will be implemented where appropriate to minimise experimental biases and all experiments will be documented and reported transparently.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Breeding colony management guidelines from NC3Rs and the Jackson Laboratory will be followed to optimise our breeding strategy, with appropriate controls to ensure the strain genetic integrity and all the appropriate records.

Most of the genetically altered animals used in this project already exist and will be obtained from the relevant suppliers or collaborators. Only in rare occasions we expect to make a required line ourselves using a transgenic company service. If feasible, CRISPR/Cas9a gene editing technology will be used to generate genetically altered mice which should reduce the number of used animals.

Transgenic mouse colonies will be maintained as homozygote whenever possible, to minimize the number of unwanted genotypes. We will keep stocks of frozen sperm and embryos in order to avoid unnecessary breeding if a line is not required for several months. Where possible transgene negative mice resulting from breeding will be included in experiments and tissue collections, to maximize the research output and reduce the number of experimental mice. Some of the used reporter mice can be genotyped immediately after birth by assessing the fluorescence transgene expression, preventing unnecessary weaning of unwanted genotypes.

Pilot animal data will be shared with other research groups (e.g., tissues) to allow optimization of specific experiments (e.g., antibody testing) without the need to sacrifice animals. Additionally, suitable publicly available data will be used and analysed to inform any of our studies (e.g., sequencing data).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice have been selected because they are a well-validated experimental model and are the lowest form of mammal that mimics key aspects of human skin physiology. This includes the change in skin cell behaviour during development, the complex cellular interplay during wound healing as well as the response of different skin cell populations to environmental stress, inflammation and disease. Mice are the only mammal in which transgene technology works reliably.

Mice are housed under barrier conditions. Most GA lines are well characterized and will not develop any harmful phenotype. Transgene activation may induce temporary discomfort and body weight loss; however, our team is well familiarized with the care and monitoring of these animals. We will aim to use refined approaches for transgene activator dosing (e.g., topical application, minimize carrier volume) of adult mice.

For monitoring skin phenotypes we will always priorities non-invasive imaging techniques such as IVIS optical imaging which have minimal adverse effects.
We have extensive prior experience of most substances we expect to administer as part of these protocols including optimal dosing strategies and information on tolerability and efficacy. Whenever any new substances are to be used, small scale pilot experiments adapted from existing data will be performed to confirm efficacy (using assays and humane endpoints appropriate to the substance) and identify any adverse effects to minimise any mild to temporarily moderate severity. Substances will always be administered in a sterile form and where possible clinical grade compounds will be used.

In accordance with the LASA guidelines for aseptic surgery aseptic techniques and sterile punches and needles will be used to minimize post-surgical infection. Measures supporting maintenance of body temperature, provision of oxygen and protecting cornea from damage by use of a protective eye ointment will contribute to overall refinement of anaesthesia where appropriate. As needed, analgesia will be provided to minimise pain and distress.

The protocols we will use to induce skin cancer are well established and characterized in the field and our lab, recapitulating key steps of human skin tumour development. Because we are aiming to focus on the early development of skin cancer, progression to severe clinical signs are not expected. However any animal that experiences discomfort as a result of the site of a tumour (e.g. around the eyes, mouth, anus, footpad), tumour burden or shows tumour ulcerations with necrotic areas resulting in long-term skin break down (non responsive to treatment >24 hours) or other impaired body conditions (e.g. persistent hypothermia, anaemia or significant abdominal distension) will be killed.

Why can’t you use animals that are less sentient?

It is essential that any GA animal model used in this programme of study reflects as closely as possible the human disease or allows us to examine the functions of genes within that disease. Thus, our choice of GA models will always follow this objective to answer relevant

questions for gaining a deeper understanding of human pathologies and develop new therapeutic strategies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

When generating transgenic mice we will use inducible transgenes whenever possible, providing us with more experimental flexibility and reducing the time window when mice display a phenotype.

All surgical procedures will be done aseptically and peri-operative analgesia will be given and maintained after surgery for as long as necessary to alleviate pain. Mouse behaviour and appearance will be carefully monitored for post-operative pain or discomfort.

For wound healing experiments we will priorities small 2mm punch biopsies which heal faster and allow to analyse more than one wound per animal, reducing the maintenance and number of experimental mice.

When applying a substance, we will follow dosing and volume recommendations from the literature and current welfare guidelines (see next section).

If a new drug is tested, an initial experiment is carried out with a small number of mice and the conditions predicted from the literature to be most effective and minimize the risk of general adverse effects. The data from the first experiment then informs the design of subsequent experiments, which may involve larger numbers of mice.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

N3CRs, including experimental designs (https://www.nc3rs.org.uk/our-portfolio/experimental-design- assistant-eda) and colony management (https://nc3rs.org.uk/3rs-resources/breeding-and-colony- management/colony-management-best-practice), PREPARE (https://norecopa.no/prepare), FRAME (https://frame.org.uk/resources/experimental-design/) and ARRIVE 2.0 for transparency in reporting (https://arriveguidelines.org/arrive-guidelines) guidelines will be used.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

To actively follow and implement the latest 3Rs advances, we will keep up to date via the NC3Rs resource library updates, UAR (Understanding Animal Research), LASA, NORECOPA, FRAME (Fund for the Replacement of Animals in Medical Experiments). We will seek other 3Rs related databases, including ALtweb (www.altweb.jhsph.edu) and CAAT (Center for Alternatives to Animal Testing; http://caat.jhsph.edu/about-caat/). We will continuously seek help by following UFAW, LASA Best Practice Guidelines series, and ECVAM (European Centre for Validation of Alternative Methods). A review of these datasets will be added to our lab meetings' agenda. We will also always engage with other project licence holders to identify novel methods and training needs.

40. The regulation and function of macrophages during health and disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Inflammation, Infection, Sex dimorphisms, Immune cells, Tissue repair

Animal types	Life stages
Mice	adult, pregnant, neonate, juvenile, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Immune cells called macrophages are central to the maintenance, defence and repair of tissues. The overall aim of this project is to understand what regulates the behaviour, number and survival of macrophages during health and disease and how these processes can be targeted to improve tissue repair and resolution of inflammatory and infectious disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Macrophages reside within all tissues of the body where they function to maintain tissue integrity through many mechanisms including protecting against infection, regulating inflammation, clearing dead, dying and damaged tissue, and regulating regeneration and scar formation following injury. Unfortunately, macrophages do not always behave in an optimal manner, leading to susceptibility to infection, excessive inflammation, and defective repair, and they may even drive facets of disease through aberrant function. The outcome of disease can also be determined by the overall number of macrophages present within a tissue, and therapeutic benefits of supplementing these cells has been

shown in numerous preclinical disease models. Hence, understanding the signals and processes that dictate the number and behaviour of macrophages within tissues may reveal novel pathways that can be targeted when these cells behave aberrantly or sub-optimally during disease.

The function of macrophages is predominantly dictated by the tissue microenvironment in which they differentiate and reside. Hence, understanding the tissue factors that programme resident macrophages in normal healthy tissues may enable us to promote or target these signals when macrophages behave aberrantly during disease. Of particular interest is a person's sex, since this is a major variable determining the risk and outcome of many inflammatory and immune-mediated diseases, and macrophages appear more highly regulated by sex than most immune cells. However, macrophage function may also be affected by factors other than their immediate microenvironment.

For example, the huge breadth of function achieved by macrophages in part arises from distinct patterns of behaviour of resident cells present within tissues during health and those recruited from the blood upon injury or disease. These distinctions can persist for many months after inflammation/disease has resolved. We have shown that even in healthy tissues, recruited cells can exhibit distinct behaviour from established resident macrophages for many months in part because these populations counter-regulate one-another through as yet undefined pathways. Hence, fully disentangling the distinct functions and regulatory pathways of these distinct populations remains paramount if we are to target particular processes controlled by these cells during disease. Emerging evidence also suggests that exposure to inflammation can change how tissues and macrophages respond to future disease events, a process termed innate tissue training. In other words, the life history of a macrophage or its microenvironment may dictate how these cells behave during subsequent stress responses. We want to understand how macrophages learn to respond differently to inflammation and injury, to what degree this alters the outcome of future disease, and whether this training can be reversed or re-programmed.

Our primary focus will be diseases of the body cavities and liver. Macrophages are critical effector cells in acute liver injury and regulate the severity of pathology in chronic liver disease. Peritoneal macrophages are essential for protection against peritoneal infection, one of the leading causes of sepsis. These cells have also been implicated in the development of abdominal adhesions, a type of fibrotic scar tissue which forms within the abdominal cavity between the surfaces of organs following injury or inflammation, most commonly abdominal surgery or peritonitis. Combined, these diseases can cause significant mortality or ongoing morbidity and hence understanding the cellular processes involved may lead to novel therapeutic development.

What outputs do you think you will see at the end of this project?

This project will address fundamental principles in macrophage biology and is therefore likely to have far reaching implications for many disease processes. As such, the primary output will be generation of new knowledge, methods and datasets that will be largely disseminated by publication in peer reviewed journals and on-line repositories, and presentation at conferences, seminars and workshops. However, our work may also generate exploitable intellectual property that could lead to patents and development of commercially exploitable immunological therapies to boost or alter the function of macrophages during disease. For example, work performed under my previous Home

Office licenses contributed to a patent granted for new therapeutic approaches to expand macrophages and their monocyte precursors during acute respiratory distress syndrome. We also expect the findings from this project will lead to addition research funding and may help us drive greater patient involvement in research.

Who or what will benefit from these outputs, and how?

The academic community will be the primary short-term beneficiary of this project. Although only a restricted number of disease models and tissues will be investigated under this license, the broad range of diseases in which macrophages play a role and the fundamental principles of macrophage biology that will be examined in this project means that the knowledge produced is likely to affect a broad section of the academic community (eg immunologists, clinicians, physiologists, and those interested in aging). The knowledge generated will also be relevant to the pathophysiology of the modelled human diseases and therefore will be of interest to clinicians who specialise in these fields.

The mechanistic knowledge gained from this project could also steer future research towards new macrophage-based therapeutic strategies and hence will be of long-term benefit to the pharmaceutical industry and ultimately to patient health. The timeframe for improvements to human health would be expected in the range of 10-20 years, while increased investment in Research and Development in these areas could occur within 3-5 years.

How will you look to maximise the outputs of this work?

Findings from this project will primarily be communicated and disseminated through publication in widely-read peer-reviewed journals, but also presentation at local, national and international congresses and individual institute seminars. To ensure maximum dissemination that includes lay- persons and patients, only journals with Plan-S compliant open access options will be considered. Furthermore, to expedite dissemination of knowledge, data will be published on an open access preprint repository such as bioRxiv. We are also supported by the University's Press and Public Relations office to ensure maximum dissemination of our research activities and findings via the media. To ensure dissemination of all new knowledge and prevent unknowing and unnecessary repetition of experiments by others, I will seek to publish all data generated under this project including negative results, again utilizing preprint repositories such as bioRxiv if necessary.

To rapidly translate our findings to the human arena I will exploit collaborations with local clinicians to access clinical samples from patients with peritoneal pathologies (eg. adhesions and accompanying peritoneal cells removed during corrective surgery) and, where possible ostensibly healthy individuals (eg peritoneal cells from patients undergoing hernia operations or elective laparotomy). For this purpose, the medical school environment at our institute is ideal for developing collaborations to assess the relevance of findings in human disease and translational research.

Research at our institute is also ideally placed for translation of our findings, with a dedicated wholly owned subsidiary in place to offer management of technology transfer, company formation and incubation facilities. Hence, this will maximise commercial outputs should the proposal yield potential opportunities beyond academia.

Species and numbers of animals expected to be used

- Mice: 10000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We study mice because their immune system, tissue physiology and development are very similar to that of humans and because scientists have created many genetically altered mouse lines that allow us to dissect in fine detail what happens during immune responses or following tissue injury. For example, we can now determine the precise function of a molecule, often in a specific cell type and even at a precise time in development or point during infection, injury or inflammation, and we can follow the fate of individual immune cells and determine how their function changes over an entire disease course to determine how they enable or hinder resolution of disease. As we wish to understand how immune cells are programmed during normal development and why this depends on if they come from the bone marrow in adult animals or were established in tissues right at beginning of development in the embryo, we need to study these cells over all development stages of an animal.

Specifically, pregnant dams are needed so we can label and track cells that develop in embryos through to adulthood; we need juveniles to determine what happens in tissues early in life that prevents new immune cells from entering from the blood, and we need to study adults to determine how these different types of immune cells function when tissues become infected or damaged. Finally, we also propose to study pregnant mice. During pregnancy many tissues undergo dramatic and rapid growth, which is accompanied by major changes in the function of adaptive and innate immune cells.

Hence, pregnancy is a natural physiological system in which to understand the cross-talk between macrophages, tissue cells and immune cells that occur during growth and involution of tissues and may facilitate these processes and changes in immune cells required to maintain foetal and maternal health.

Typically, what will be done to an animal used in your project?

Some mice will be used for breeding and colony maintenance only.

Many experiments start with a step to track specific types of immune cells, chiefly macrophages. This will include in embryos in utero by administration of substances to pregnant dams, or by tracking development of cells in juveniles or adults by administration of substances or cells at these different life stages. For this purpose, some mice may be exposed to radiation to allow us to identify and study cells that come from the bone marrow. We will then assess the fate and function of these immune cells over the course of development in healthy tissue or following pregnancy, providing us with a baseline from which to understand what are ‘normal’ and ‘healthy’ functions and how the number and function of these cells is regulated during health. To understand what signals dictate these normal healthy functions and how these change with disease, we will then manipulate the tissue environment by depleting specific types of tissue/immune cells, blocking signalling

pathways by giving drugs, or manipulating expression of genes in specific cell types using genetically altered animals, or by inducing acute or chronic chemical injury in the liver or physical, inflammatory or infectious injury in the peritoneal cavity by surgery or administration of inflammatory compounds or infectious agents by intraperitoneal injection. As we are particularly interested in how the sex of animal effects the function of macrophages, some animals will have sex organs removed surgically, or signalling through hormone receptors blocked pharmacologically or using genetically-altered animals.

To establish the long-term effect of altering the tissue environment or of inflammation or injury on the normal protective function of tissue macrophages, some animals that have received modifications to their tissue environments or an initial episode of inflammation or injury, will be allowed to recover for a period of months before receiving a subsequent bacterial infection or repeat episode of tissue injury or inflammation.

When performing so called rescue-of-function experiments where we set out to prove that a cell type is required for normal function of our immune cells is needed because it produces a factor X, we will both deplete a cell type and then “rescue” the effect of cell loss by administering mice with factor X in drug form, before subsequently infecting or causing tissue injury.

In some experiments, small volumes of blood may be taken usually from the tail vein, for example to screen for efficacy of cell labelling or depletion techniques. Finally, some experiments may end with animals being killed under terminal anaesthesia to enable removal of large volumes of blood or allow fixation of tissues.

What are the expected impacts and/or adverse effects for the animals during your project?

No adverse or harmful phenotypes are expected from breeding and maintenance of genetically modified mice. Mice will be monitored for ill health and will be humanely killed if a humane endpoint is reached.

Acute liver injury can lead to a short period (6-12 hrs) of moderate suffering and discomfort evidenced by reduced activity and reduced food and drink consumption but from which animals quickly recover.

Chronic liver injury is generally well-tolerated as lower doses of chemical toxins are used. Each bi- weekly injection of toxin can induce periods of transient (<30mins) lack of activity indicative of general discomfort while chronic injury can lead to sustained mild (<10%) to moderate (<20%) weight loss. A small number of animals may approach 20% weight loss, and/or exhibit hunched posture, pale skin, and decreased activity/mobility, indicative of the upper limit of the moderate pain and discomfort threshold and if so, will be removed from the study and killed.

Sterile inflammation in the body cavities is generally well tolerated and does not result in clinical signs of discomfort or pain. However, injection of some bacterial components that induce inflammation can lead to transient loss of body temperature (12-24hrs), which will be counteracted by providing animals with heat support.

The time course of intravenous infection with bacteria will be limited to 30 mins to ensure animals are killed before they experience more than mild discomfort.

Surgical procedures including laparotomy, stimulation of adhesion formation, removal of ovaries or testes is performed under general anaesthesia and is accompanied by delivery of appropriate post- surgical analgesia for pain relief to ensure animals do not experience more than a moderate degree of pain and discomfort.

Most animals exposed to radiation will receive body protection and therefore will experience only mild adverse effects associated with general anaesthesia rather than exhibiting outward clinical signs.

Exposure to lethal whole-body levels of ionising radiation leads to elevated risk of bacterial infection, diarrhoea, and signs of general discomfort for a period of several days. These impacts are controlled by keeping animals on water containing antibiotics for 1 month post irradiation and supplementing their diet with easy-to-eat food.

Delivery of most substances that interfere with cell signalling, cause deletion or upregulation of gene expression or depletion of cells does not in general lead to more than transient distress associated with route of delivery, or mild irritation at the injection site.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Approximately 40% of animals will be used for establishment and maintenance of genetically altered animal breeding programmes or for production of genetically altered mice that will simply be used for harvesting tissues. These animals will fall will into a subthreshold limit of severity. Of the remainder, approximately half of animals will experience degrees of suffering that fall within a moderate severity category, including induction of sterile and bacterial peritonitis/pleuritis, surgery, acute liver injury, chronic liver fibrosis, and exposure to irradiation, while the remainder will experience degrees of suffering that fall within mild severity including induction of short-term (<30min) intravenous bacterial infection, adoptive transfer of cells, and substance-mediated induction or deletion of gene expression and cell depletion strategies. Animals killed under terminal anaesthesia for extraction of blood or fixation of tissues but not experiencing any other procedure while alive will be classed as non-recovery but will be relatively few in number.

What will happen to animals used in this project?

- Used in other projects
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The immune system and most disease processes rely upon a complex series of spatially and temporally segregated interactions between cells of diverse function and origin. To understand such complexity, it is essential to undertake research in vivo, since this cannot yet be modelled meaningfully in vitro.

Which non-animal alternatives did you consider for use in this project?

1) Analysis of human tissues and cells.
2) Use of cell lines.
3) In vitro organoids.

Why were they not suitable?

The types of analysis required to track cells in the body to determine how the life history of cell determines its function are not possible by simple analysis of human tissue biopsies. Although a limited assessment of whether immune cells are generated from the tissue or bone marrow can be made from biopsies of patients receiving sex-mismatched tissue or BM transplants, access to these kinds of samples is difficult due to scarcity and ethics prevent removal of cells or biopsies without good clinical reason or in sufficient quantities to allow in vitro analysis of cell function. In addition, a main objective of this project is to understand how and why macrophages behave a certain way in healthy tissues, as this will provide a baseline from which we can fully understand pathological processes that occur in diseased tissues and determine what features need to be reinstated to enable tissues to return to a resting healthy state. Again, ethical considerations largely prevent the removal of healthy tissues from humans for this purpose. Hence, our analysis of human tissue will largely be restricted to the context of disease or following disease resolution.

Many functions of immune cells are imprinted by their immediate tissue environment and these are lost rapidly after they are removed from the tissue. This largely precludes the use of cell lines for our work.

Hence, while studies of the micro-environmental factors that control and imprint tissue-specific macrophage features will be performed in part using mixed cell cultures or organoids in vitro, this methodology will still require primary cells from animals, culture supernatants of primary cells, or slices of primary tissues, together with validation of results using in vivo methodology.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals has been estimated based on experience gained under my previous two Home Office licenses, my current and predicted levels of funding, and my planned experiments for the next 5 years. In this regard, the work plan and nature of

experiments covered by this project are similar in design to those covered under my previous licenses.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

For all of our experiments in-bred mice are used to reduce intra-mouse variation. We also generally use a randomized block design for experiments to further reduce factors that could cause differences between animals that are unrelated to factors being tested in experiments (eg cage to cage variation). By reducing natural differences between animals in this way, we reduce the number of animals needed to identify differences caused our experimental test. In cases where we cannot use randomized blocking to reduce cage effects, we will still ensure that animals are littermate-controlled to reduce variations that can arise between litters. Our experiments are also designed to reduce the number of variables to as few as possible and thereby the number of control groups required. In this respect, we will always consider carefully whether it is important to include 'naïve' as well as 'vehicle' control groups in experiments, or if the latter alone is sufficient for interpretation of results.

To ensure best practice in statistical analysis and experimental design all new staff members working under this license will undergo training by the license holder or another experienced researcher and experimental planning will be undertaken using the NC3Rs EDA and the incorporated power calculator. Planned experiments are discussed regularly within group meetings to ensure all are correctly controlled and to facilitate sharing of tissues/data for the most effective use of animals.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will perform pilot experiments when investigating new interventions to determine selection of optimal parameters such as dose and timepoints. We will use animal colony management software (tick@lab) to enable efficient breeding of mouse lines and lines will be cryopreserved once work is finished. Planned experiments will be discussed regularly within group meetings to facilitate sharing of tissues/data for the most effective use of animals. Furthermore, tissue and data that we generate will be shared with the scientific community to maximise this resource and if possible, we will make use of archived tissue from collaborators. When investigating new genes of interest we will, whenever possible, first import bone-marrow from genetically-altered mouse lines and test for function in irradiated mice before deciding whether rederivation into our animal units is required, thereby saving considerably on animals used for rederivation and breeding.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice represent the most tractable model for the in vivo study of macrophage biology given the availability of genetically modified strains and commercial reagents required to effectively determine cell origins, lifespan and the function of individual genes within defined cell subsets. Furthermore, mice are an appropriate model since they reproduce many features of the human immune system relevant to this project.

I am committed to ensuring the most refined protocols are used in our studies. To this end, my group has pioneered the use of a method to identify and track bone marrow-derived cells and determine the lifespan of tissue resident macrophages that involves irradiation with lead shielding to protect the large proportion of animal tissues. This represents a significant refinement to studies that use whole body irradiation or parabiosis. While we still employ whole body irradiation without shielding in some circumstances, this is atypical and we ensure that the irradiation is delivered in split doses as this is better tolerated by animals. In a further refinement, we have developed a system to adoptively transfer and track mature immune cells thereby by-passing the need entirely for irradiation in a significant proportion of our work. Where possible, we will also use tamoxifen-inducible genetic systems for fate- mapping, to complement these studies while causing minimal harms. Existing knockout and transgenic mouse lines (e.g. Cre/LoxP lines and fluorescent reporter lines) will allow testing of specific mechanistic hypotheses, and may be used together with bone marrow chimeric approaches to further refine pathways of cellular communication.

To study the long-term effects of local inflammation on function of peritoneal macrophages, our first approach will be to use sterile models of inflammation since these are accompanied by very limited clinical signs. While we will also determine the effect of surgical injury/adhesions on long term function of peritoneal macrophages, we will dissect the mechanisms underlying common effects of surgery and sterile inflammation using the less harmful sterile models. Blood and local bacterial infections will be used to determine the effect of tissue factors, sex or previous inflammatory events on the function of tissue macrophages in local immune defence. We will primarily use infection models that are well tolerated (eg attenuated Salmonella strains), but will also use more virulent infection models but purposefully choose early endpoints to assess the ability of animals to contain infection but before development of severe levels of harm.

To study the mechanisms of post-operative adhesion development, we will use the most reliable and reproducible method of adhesion formation to reduce animal use and cause minimal harms.

Models of acute and chronic liver injury will also be used. Acute injury is generally well tolerated in mice and induces only transient suffering at the low doses used by my group. The model of chronic liver injury that we will use is more controllable and predictable than other such models and is also reversible, meaning animals quickly recover following cessation of injury. Although this chronic injury model is commonly studied over a period of 12 weeks, we have found that even only 4 weeks of injury leads to a long-term effects on liver macrophage function that persist following recovery. For this reason, in most experiments we will purposefully use this shorter time frame to reduce suffering.

Why can't you use animals that are less sentient?

A mammalian model is required to study an immune system with a high degree of functional, anatomical and developmental similarity to humans. The study of inflammation and disease models requires the use of adult mice because inflammation and tissue injury is altered in juvenile life stages. The duration of the homeostatic and pathological processes to be studied prevent using mice under terminal anaesthetic.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In line with local policy, we have adopted the latest techniques in animal handling (eg cupping) to significantly reduce the stress associated with procedures. Furthermore, where possible, the least invasive methods for dosing and sampling will be applied. For example, we now only use oral dosing for delivery of tamoxifen. Anaesthesia and analgesia will be provided where suitable (eg for humane restraint, during and recovery from surgery). Animals will be routinely checked following procedures to assess for pain or discomfort, and animals will be humanely culled if any health issues arise that cannot be immediately treated. To reduce infection risk, the best aseptic technique will be used during surgery (eg sterilization of instruments between animals, full surgical drapes) and immunocompromised mice will be housed in IVC cages. Infection experiments with either utilize attenuated organisms, or early endpoints will be used that prevent animals experiencing more severe harms. Where possible, we will use genetically altered mice in which genes are deleted from specific cells types to reduce the possibility of off-target harms of using animals with pan-cell deficiencies.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Our institute employs a dedicated team of veterinarians that are continually seeking to improve animal welfare and refine animal use. My group consults closely with this team and takes full advantage of the extensive resources provided on their website to ensure we are following current best practices. These resources include comprehensive guidelines and standard operating procedures for most common rodent procedures. We also regularly consult the NC3R's resource library for up to date best-practice guidance.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our institute employs a team of dedicated veterinarians that are continually seeking to improve animal welfare and refine animal use. My group consult closely with this team and take full advantage of the extensive resources provided on their website to ensure we are following current best practices to minimise pain, distress and lasting harms to the animals. These resources include comprehensive guidelines and standard operating procedures for most common rodent procedures that are continually being updated. We will also take full advantage of the annual 3R's seminar day and roadshows organised by our Establishment and 3R's and Culture of Care Committee to find out about pioneering developments in best practice, and will continue to receive and engage in the NC3Rs newsletter.

41. Epigenetic gene regulation in ageing and rejuvenation

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Ageing, Reprogramming, Rejuvenation, Cell health, Resilience

Animal types	Life stages
Mice	juvenile, adult, embryo, neonate, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this programme of work is to study how age-associated epigenetic changes affect health and life span. Ultimately we want to reprogramme or rejuvenate these epigenetic processes to help reverse disease and improve healthspan.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Age related conditions and disease impose a significant rising cost to society as globally ageing populations increase. It has become pertinent to investigate how, during this

greater lifespan, we can enjoy better health. Our experimental work will give novel insights into epigenetic reprogramming and rejuvenation mechanisms that regulate associated changes in cellular states, with the aim of identifying interventions that slow, prevent, or reverse these disease-related states.

What outputs do you think you will see at the end of this project?

We hope to see scientific progress in key areas of our inquiry, which would translate into peer reviewed publications and intellectual property together with its initial translation or licensing.
For commercially sensitive applications, these will also be associated with development of patents that lead to treatment of disease states with the ultimate aim to extend healthspan. The part of your life when you are healthy and free from serious disease or disabilities, is described as your healthspan.

We aim to discover novel epigenetic rejuvenation factors, including DNA repair factors, transcription factors, and DNA methylation and chromatin modulators. This will eventually lead to the discovery of new targets and drugs, to treat various diseases that occur throughout the lifespan. One of our models is regeneration of the liver where the capacity to do so declines with age.

Who or what will benefit from these outputs, and how?

In the short-term (2-5 years):

The scientific community will benefit from an increased knowledge of basic science in the fields of epigenetic reprogramming from early development in embryos, rejuvenation in somatic cells and in organisms. In the commercial sector, our company and other companies with interests in epigenetic processes as drug targets or biomarkers will benefit.

In the longer-term (5-20 years):

We hope that society at large should benefit from our discoveries as our conceptual academic research is validated in further preclinical animal models, in human clinical studies, and where possible provide a broad impact on human healthcare. This will be done within our company or in partnership with other companies or contract research organisations (CROs).

How will you look to maximise the outputs of this work?

We are putting together larger collaborative teams across our company to accelerate progress and dissemination of new knowledge. This includes the collection of metadata across the institution. We mandate the use of electronic notebooks, for registered tissue entries and all work documentation, which is open to other scientists in the company in order to avoid duplication.

When appropriate we will publish results (positive or negative) and we will file patents.

Currently there are no outreach plans.

Species and numbers of animals expected to be used

- Mice: 39440

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mouse models play an essential role in preclinical and translational research. There are also large datasets created from mice available to advance the study of reprogramming and ageing. We will employ both wild-type mice and genetically engineered mouse models to look at epigenetic reprogramming during embryonic development and the full lifecourse and in a variety of tissues pertinent to our scientific aims.
To be able to collect early embryos it is effective to use juvenile stages of females rather than their adult counterparts to produce a larger number of oocytes and zygotes thus reducing the overall numbers of mice required.
Adult males and females are required for breeding, embryo rederivation (pseudo-pregnant females) and cryopreservation.

Adult and aged male and female mice are required to measure cellular epigenetic changes during ageing that may underlie progression of frailty and age-associated diseases.

Typically, what will be done to an animal used in your project?

Breeding and maintenance to produce juvenile, adult, pregnant or aged mice that will be culled to supply tissues for the aims described in this project.

We will continue to use genetically altered mouse strains, that have been generated under our current project license, which incorporates a highly refined model system that can provide robust targeted protein degradation which acts in a rapid, inducible, and reversible manner.

We shall also produce gene inducible mice controlled by drugs to help modify gene expression patterns in their cells to trigger epigenetic pathways.

We will use drugs to enable, facilitate, or inhibit cellular reprogramming and disease as a function of age. Especially useful on studies with ageing disease models.
We will induce acute liver damage to understand the effects on cell populations during recovery and repair.

Female mice will be superovulated and then culled to provide materials to establish the expression of reprogramming factors during early embryonic development and to derive genetically altered mouse strains.

Mice will undergo surgery for embryo transfer, vasectomy or subcutaneous implants, the duration of anaesthesia and surgery are short.

What are the expected impacts and/or adverse effects for the animals during your project?

The general type of genetically altered mice produced under the breeding and maintenance protocol (mild) in this project are either conditional knockouts, or drug-inducible transgenics or knockouts, or knockdowns of protein expression. All require the presence of a substance activator and as such is time limited and also in the case of the protein knockdown reversible. This avoids using the whole animal where constitutive ablation could have a severe phenotype. This general type of technology is more controllable and thus helps avoid adverse effects. Depending on the specific gene, once induced, the mice may exhibit weigh loss, hair loss and or behavioural stress.
We will also use mice under our breeding and maintenance protocol (moderate) in this project that exhibit genetic disease so that we can introduce interventions to promote efficient repair or rejuvenation.

For mice that undergo surgery for embryo transfer or vasectomy, subcutaneous implants, the duration of anaesthesia and surgery is short and the animals are expected to make a full and unremarkable recovery, although they will be administered analgesia to mitigate against short-lived pain.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Sub-Threshold 50%
Mild-25%
Moderate 25%

What will happen to animals used in this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We do many experiments without using animal models however, the interactions of different cellular populations within a tissue or an organism are currently too complex to recapitulate in a culture dish; for this reason it is essential to carry out some experiments on animals. This is particularly true in the context of fetal development and ageing models where culturing conditions are still too limited.

Which non-animal alternatives did you consider for use in this project?

- Embryonic stem cell based systems:

- for the study of epigenetic reprogramming in early embryos
- devising cells in a culture dish that resemble early embryo stages to mimic the development of early germ cells
- to study reprogramming at and after embryo implantation Adult cell based systems:
- for characterising epigenetic changes in ageing for reprogramming and rejuvenation
- modelling via computational predictors

Why were they not suitable?

Each of the models described above has advantages over animal models including a reduced financial and time cost, as well as an ethical benefit. However, these models are limited in their ability to fully recapitulate tissue states and the interactions between tissues, particularly during ageing.

Additionally, models that require developmental processes and reprogramming (cell culture or organoid models) do not fully recapitulate in-vivo embryonic dynamics.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers are based on previous experience of the breeding strategies involved with maintaining the different transgenic mouse colonies we produce (which can involve multiple alleles together in a single animal).
Small pilot studies that provide a proof of concept give a good estimate of quantities for scaled up experimental cohorts required to supply statistically sound results.
Many of our standard protocols have a known output with wild type strains used for controls. These wild types are often the base background used to create our GM strains, so, quite often, the number requirements are similar.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have been able to reduce the number of animals used through dramatic improvements in single cell technology, which look at multiple layers of epigenetic regulation; current protocols are designed to enable us to obtain as much information as we can utilizing this.
We have accessed the NC3R's Experimental Design Assistant along with the ARRIVE guidelines for estimating numbers required for new protocols, in conjunction with the methods from peer reviewed publications.
Online tools e.g., G*Power are available for Power Calculation, and we will consult biostatisticians in designing animal experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Our institution has implemented many tools for rapid collaborative in-company experimental data/tissue sharing. For example our in-vivo hub supply a weekly list of what post cull tissue is available in excess of the prime purpose for that animal. This has been championed in our institute and has greatly reduced animal usage. This is now a regular schedule of our inhouse culture.
We will conduct small pilot studies which will be used to calculate the minimum number of animals necessary to obtain statistically relevant results, which will enable us to correctly power the experiment with the input of our in-house Bioinformaticians and Machine Learning group.

We will also collect and store as many samples as we can from each experiment.This will help us build a library of tissues we can use in future research. This not only reduces the number of new experiments we need to run, but also allows us to share material with other researchers.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We plan to use mouse GM strains that utilise a system of targeted protein degradation which acts in a rapid, inducible, and reversible manner. This means the mice show no phenotype and breed normally when uninduced. As a result the animals will suffer no pain or distress during their normal lifespan until being used.
Progeria mice model: Progeria is a human disease of accelerated ageing. We need to use these mice, which have symptoms similar to this disease, to understand how we can use epigenetic interventions with the aim to alleviate this condition. These mice will be closely monitored, and humane endpoints applied to make sure any suffering can be avoided where possible.

Yamanaka Factor Inducible models: These drug inducible models provide tissue specific (e.g., just the liver) or whole body activation of genes known as the Yamanaka factors. In these models the mice show varying effect in relation to increase in drug dose, however they show no phenotype and breed normally when uninduced. As a result the animals will suffer no pain or distress during their normal lifespan until being used.
Naturally aged mice are required to allow for characterisation of the epigenetic changes and possible reversal after treatment. These mice will be closely monitored, and humane endpoints applied to make sure any suffering can be avoided where possible.

Tissue damage in the liver will be used to study the regulation of changes during ageing. We will use these mice to understand the long-term effects on cell populations and organ health and challenge the damage with rejuvenation factors to see if we can rescue age-associated changes. Our work is not driven by clinical endpoints; we do not intend to continue until the mice develop liver failure.Therefore, in most cases mice will have no signs, but we are aware of the possibility that in some cases liver disease may progress more quickly and the mice develop adverse clinical signs. These mice will be closely monitored, and humane endpoints applied to make sure any suffering can be avoided where possible.

Why can't you use animals that are less sentient?

We want to transfer our findings to human biology in order to contribute to diagnostic and therapeutic approaches in humans.
Global epigenetic reprogramming has only been described in mammals.
Important aspects of cell lineage decision from early embryos, to tissues and organs can only be fully studied in mammals.
For the study of tissue damage, ageing, and cell fate we require the use of mammalian organisms with fully developed organs.
The most reliable ageing clocks have only been described in mammals and need to work across the whole ageing spectrum.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Refined aseptic surgical technique that the Named Vet has to sign off for competency.
Animals approaching the agreed limit of severity are checked more regularly and humanely killed if they are likely to exceed the severity limit. This is monitored by inhouse NACWOs.

We have invested in new animal models using advanced refined model systems. This means the mice show no phenotype and breed normally when uninduced.
Effective colony management.

For our animals held under moderate breeding protocol, we are using well defined disease models, with known conditions that have set parameters for endpoints.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

When planning experiments, we will refer to the PREPARE guidelines and for general guidance in our experiments we adhere to the LASA guidelines.
For ageing studies, we will utilise best practice guidelines such as: "A toolbox for the longitudinal assessment of healthspan in ageing mice" (Bellantuono I. et al. Nat Protoc. 2020)," Aging Research Using Mouse Models" (Ackert-Bicknell CL et al. Curr. Protoc. Mouse Biol. 2015), and "A

Clinical frailty index in ageing mice: comparisons with frailty index in humans" (Whitehead et al. J. Gerontol. A Biol. Sci. Med. Sci. 2014).

We will use the NC3Rs guidelines on breeding to minimise wastage as well as conforming to the home office “Efficient breeding of genetically altered animals assessment framework” to ensure the best practice when maintaining colonies.
We will use online tools e.g., G*Power are available for Power Calculation, and we will consult biostatisticians in designing animal experiments.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We receive regular updates from the NC3Rs newsletter and will have regular discussions with the Named Persons at our facility as well as attending regular in-vivo working groups to keep abreast of any major updates that may impact our work.
Where new protocols or refinements are reported to offer superior advancements in the field of the 3Rs we will aim to implement them within our own studies as long as this does not invalidate the results of an already established technique. In this scenario once the existing project is completed, any new projects that require the same technique would implement the new protocols. This will keep the controls pertinent to the work being carried out and not switched halfway through.
We will also attend conferences to engage in proactive discussions with groups performing experiments in the same or related fields both locally and in geographically distant locations. Sharing of experiences and protocols is essential to ensure achievement of good scientific data with fewer mice using refined techniques and technology.

42. Early-Stage Parkinson’s disease: understanding changes in the brain and testing new therapies.

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Parkinson's disease, Neurodegenerative disease, Gut-brain axis, Therapy

Animal types	Life stages
Rats	adult
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the project is to study the changes that occur in the brain of a rodent model of early stage Parkinson’s disease and to test possible treatments to prevent these changes and potentially slow down the progression of the disease.
Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Little is known about what causes some of the early signs of Parkinson’s disease, like the loss of smell, memory loss or changes in the microorganisms in the gut. However, it is crucial to understand the causes of these signs to be able to develop better treatments. These symptoms can happen more than 10 years before a diagnosis is made based on

the start of the typical tremor and slowness of movements seen in Parkinson's disease. Detection of the early changes in the brain may improve diagnostics and new treatments for the disease.

What outputs do you think you will see at the end of this project?

Parkinson's disease, like other age-related neurodegenerative diseases, is increasing within the population as the average age increases. The number of people diagnosed in the UK is about 145,000 (around 1 adult in every 350). Current Parkinson's disease treatments are only targeting the symptoms but fail to stop the progression of the disease. In order to find new treatments, we need to determine the early cause of the disease. In this project, we aim to collect new information on the changes that occur in the brains and the gut of appropriate rodent models of Parkinson's disease and test new treatments that will rely on stopping disease progression or using the capacity of the brain for self- repair. All data will be presented at national and international conferences and published in scientific journals.

Who or what will benefit from these outputs, and how?

- Scientists/doctors studying the origin of Parkinson's disease- The most effective clinical advance outside an outright cure for the disease may be an early diagnosis. Detection of early symptoms, like the loss of smell for example, in early-stage Parkinson's patients may greatly help such a diagnosis and allow treatments to prevent the death of nerve cells and stop the appearance of the more severe symptoms. To this end, appropriate rodent models that display symptoms and pathologies actually seen in patients will be a benefit to scientists and doctors who are trying to understand the origin of the disease.

- Patients suffering from Parkinson's disease- The study of models of early-stage Parkinson's disease that present symptoms similar to those displayed by Parkinson's disease patients may play a vital role in understanding the origin of the disease and determining promising treatments. Furthermore, it is vital to determine if some of the new therapies to be tested can also be effective in decreasing the symptoms observed in patients long before the typical shaking and slowed movements. The clear rescue effect of some new treatments in rodent models represents a very promising avenue for a new and long-overdue effective therapy for Parkinson's disease.
- Scientists/doctors studying other diseases caused by death of brain cells- Patients suffering from these diseases- Information from this project could also prove very useful to scientists studying the causes and treatments of other serious disorders that affect brain function such as Alzheimer's disease or multiple sclerosis that share loss of smell as a common early symptom with Parkinson's disease.

How will you look to maximise the outputs of this work?

All data will be presented in national and international conferences, discussed with experts in the Parkinson's disease field and published in scientific journals. Follow-up funding based on the data collected will be sought.

Species and numbers of animals expected to be used

- Mice: 300

- Rats: 680

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Previous scientific studies have shown that it is possible to produce models of Parkinson's disease (PD) in rats and mice that display similar symptoms than those observed in patients suffering from the disease. Young adult rodents will be used for this study to avoid undesirable side effects of the injected toxins in younger animals. Rats are the most commonly used animal in PD research and are a reference point to help compare results with previously published data. We will also establish the early stage model in mice to allow comparison with available genetically modified mouse models.
The duration of the experiments will depend on the different objectives of the project. The majority of the experiments will be terminated after 4 weeks. However, some will be continued until the rodents start experiencing some motor symptoms. A close monitoring will confirm the beginning of the symptoms and will dictate the end of the study.

Typically, what will be done to an animal used in your project?

Animals will be subjected to injections and surgical procedures via injection of a placebo solution, a toxin or a virus directly into the brain to induce the model of early-stage Parkinson's disease (PD). Behavioural experiments will be carried out at different relevant time points of the project to study both the effect of the injections and surgery (early-stage PD model) and the effects of new therapies that will be either given orally, injected or surgically administered as a gene therapy.

What are the expected impacts and/or adverse effects for the animals during your project?

It is important to consider that our intention is to look at early-stage Parkinson's disease. As such, the changes that are likely to be introduced will almost certainly be mild. To this end, we will be looking to use minimally effective doses of toxins that give a cumulative but still subtle effect in the animals tested. Animals may suffer from weight loss for a few days after the surgery. We do not expect abnormal behaviours from rodents during the project. However, animals may start moving slowly and experiencing some motor disruptions in longer experiments that will dictate the end of the experiment.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

This study will be of moderate severity due to the surgical procedures. We expect some variability in the speed of recovery between animals. However, based on past experience, we do not expect animals to show any adverse effects 3 days after surgery.

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animals will be used in this project to answer important scientific questions related to human health. The lack of cure for Parkinson's disease or even the lack of treatments aiming to slow down the progression of the disease have meant that the study of animal models mimicking the early stage of the disease may bring new therapeutic targets to light and therefore new drug treatments for the condition. The relationship between the role and functions of specific cells in the brain, their response to treatment and brain anatomy can only be explored in living tissue. This will allow existing and new data to be linked, compared directly and the more complete picture that emerges to contribute to models of brain cells and circuits in both healthy and diseased states. These models/circuits can provide invaluable insight when the questions addressed become too complex.

Which non-animal alternatives did you consider for use in this project?

All our objectives are based on the study of the aetiology of non-motor symptoms of Parkinson's disease and full or partial replacement would not be possible to mimic all aspects of Parkinson's disease with a view to test new therapies. However, the use of model systems based on patient- derived Induced pluripotent stem cells (IPC) models and organoids are being considered to partially answer some of the questions set on this project. Their use will help understand the mechanism of action of a new therapy at a cellular level and possibly model interactions between organoids and immune cells but will only give limited information as these does not fully recapitulate the complexity of the fully formed brain at specific stage of the disease. The use of animal models is ultimately essential to characterise neuronal changes in different parts of the brain, to define the causal interrelationships between regions and between body systems and determine the involvement of neuroinflammation and modulation of the observed changes following therapeutic treatment associated with behavioural changes.

Why were they not suitable?

The potential impact, both in basic research and medicine development, of model systems based on human-derived neuronal tissue that is able to transform into a cell type of interest is substantial and cultures of immortal cell lines may be used in parallel to better understand the aetiology of the disease and test new therapeutic targets. However, the mammalian brain is almost certainly the most complex biological system that exists, and cell cultures do not replicate the entire biological system and will only give limited information. Parkinson's disease is the result of disruption of several chemicals that have a unique but interlinked distribution in the brain and replicating this in a dish would not be possible.

Furthermore, the interaction between genetic mutations and toxins are also difficult to reproduce in non-animal alternatives. The characterisation of neuronal changes and modulation of these changes following therapeutic treatment associated with some change in behaviour will ultimately require the use of animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals needed for this project was estimated using previous knowledge and experience in neuroscience research. Previous experiments allowed us to predict the amount of variability between experiments and to estimate the minimal number of animals that will be required to obtain a clear answer to the research question/objective using power calculations. Typically, each animal used will provide a large amount of data towards more than one objective.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Power calculations were used to predict the number of animals needed to achieve all the objectives on this licence. Advice taken from local statisticians and the use of the results from preliminary experiments obtained on another licence helped in the design of future experiments. The amount of data/information gathered per animal in one single experiment will be maximised in order to reduce the number of animals to be used in this project and answer all the questions set in each objective.
Negative results will also be reported to prevent unnecessary studies to be conducted by other laboratories. Allocation to treatment groups will be randomised and all experiments and analyses will be blinded to maximise the statistical power of each animal used in the study.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Training of personel will be compulsory for all the techniques used in this project to ensure good practice and reproducibility of the results. Tissues (i.e. brains from all experimental groups) will be shared, increasing the amount of data collected from each experiment. Pilot studies with few animals will be carried out for each new drug to be tested to ensure that the best dose regimen is used and animal welfare is appropriately addressed.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Our intention is to induce an early stage model of Parkinson's disease in both rats and mice to study the early causes of the disease in the brain and to test new treatments. These models will therefore not suffer from the more severe side effects associated with later stages of the disease as they will be culled either before the appearance of the motor symptoms or when these symptoms are just starting to show. We have extensive experience in the method used for the induction of the models and staff will follow appropriate guidelines to perform the surgeries on these animals. Daily monitoring after the surgery will be carried out to assess the behaviour of the animals and minimise pain, discomfort and stress during the experiments.

Why can't you use animals that are less sentient?

Most patients suffering from Parkinson's disease start developing symptoms at the age of 50 or over and it is rare that patients are diagnosed with the disease when they are under 40. We cannot therefore use animals at a more immature stage of life if we are to study the aetiology of the disease. The induction of an appropriate model of Parkinson's disease requires the use of adult rodents in which the loss of the nerve cells and appearance of relevant symptoms mimic what is seen in patients.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We consider the welfare of the animals of paramount importance. The post-operative surgical care will help prevent and minimise complications related to anaesthesia and surgical procedures. We do not expect complications following the procedures in this project. However, animals will be given recovery gel (DietGel recovery), a nutritionally fortified water that aids with post-surgical recovery, and the use of analgesics will be considered pre-emptively and/or as needed after the surgery to minimise pain following close monitoring of the animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will ensure that experiments are conducted in the most refined way by following standard operating procedures from the Home Office, the LASA 2017 Guiding Principles for Preparing for and Undertaking Aseptic Surgery (E Lilleyand M. Berdoy eds.) and the guidelines for the ethical treatment of non-human animals in behavioural research and teaching [Animal Behaviour (2023) https://doi.org/10.1016/j.anbehav.2022.09.006].

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

My link to the NC3Rs through the newsletter and social media allows me to keep up-to-date with any advances in this domain. I am an active member of a local AWERB, 3Rs group, Animal Welfare Committee and London AWERB Hub and will remain informed about any updates through these roles. Any new development of new 3Rs technologies related to this project will be scrutinised and implemented if possible.

43. Development of advanced pre-clinical models for the study of tumour and host immune cell interactions and assessment of experimental therapeutics

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

cancer, immune modulation, immunotherapy, immune system, antibody

Animal types	Life stages
Mice	neonate, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to generate advanced mouse models to explore the utility of new anti- cancer reagents for use in the clinic.
The specific objectives are:
1. To generate 'near-patient' mouse models.
2. To characterise new anti-cancer reagents.
3. To determine how these reagents work and how they can be improved.
4. To develop strategies to promote/modulate the immune response to cancer and to understand how this happens.
5. To understand how tumours form with the aim of developing reagents that can stop them.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cancer is the cause of one in four of all deaths in the UK with a greater proportion still affected by cancer during their lifetime. Despite advances in screening and diagnosis cancer incidence continues to rise, largely as a result of an aging population and the impact of our modern lifestyle. The majority of cancers are diagnosed late and treatment frequently involves surgery, accompanied by chemotherapy or radiotherapy. Despite these invasive and toxic interventions patients often relapse due to the survival of small numbers of tumour cells. In spite of decades of work on treatment regimens, the survival for many cancers remained unchanged until very recently. Immunotherapy, whereby the power of the patient’s own immune system is harnessed to eradicate cancer cells, is an attractive adjunct to current treatments as it offers the possibility to be administered with maximal specificity, minimal toxicity and provide long term immune protection against recurrence. Indeed, immunotherapy has transformed the outcome of some cancer patients but these responses are variable and frequently limited to a minority of patients in a restricted number of malignancies. Clearly there is a need to develop new therapies for use either as an alternative to, or in combination with, conventional treatments.

What outputs do you think you will see at the end of this project?

We expect to be able to advance the preclinical models used for pre-clinical studies and evaluation of experimental agents. Moreover, our studies are expected to identify lead clinical candidates for first-in- human trials. We will make all resultant models and data available to the wider research community in accordance with our institutional policies.

Who or what will benefit from these outputs, and how?

In shorter term, our results will be benefit the scientific community and will help advance R&D of immune- and cancer-targeted therapies, with a longer view to translation into clinic. Once fully tested and validated (~5 years), our findings are likely to directly benefit patients in the clinic, ie, via identification of lead therapeutic candidates to enter clinical trials.

How will you look to maximise the outputs of this work?

We will make all resultant models and data (including unsuccessful approaches) available to the wider research community in accordance with our institutional policies via different channels, such as peer- reviewed open-access journals, scientific conferences, professional social media outlets (eg, LinkedIn). Additionally, we will share our experience and expertise (eg, sharing established protocols for humanisation) with those collaborators who may interested to set up such models at their own facilities, and will provide hands-on-training, where needed.

Species and numbers of animals expected to be used

- Mice: 5000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Whilst all attempts are made to reduce the use of animals by using in vitro methodology (in vitro experiments on human and mouse immune cells, explant studies (tissue grown in culture medium out of the body and organotypic models), it is inevitable in work of this nature that in vivo investigation must be undertaken. The animal models detailed are critical to facilitate in vivo proof of concept and efficacy in an amenable and manipulable system, not possible in humans. We are investigating the in vivo effects and interactions between various arms of the immune system, with a view to therapeutic application in humans. These interactions occur between different tissues and organs, and as such they cannot be fully reproduced in vitro and so systemic in vivo studies remain fundamental to the study of complex tumour microenvironments, host factors (eg, the immune system) and therapeutics. Further, advances, such as the availability of genetically altered mice and the development of more specific and sensitive techniques and reagents, continually permit a refinement and reduction in the types of experiments and the numbers of animals that are required (eg, as highlighted by the organisation 'Fund for the Replacement of Animals in Medical Experiments', http://www.frame.org.uk) and this is something to which we remain committed. The increasing availability of genetically altered mice has allowed genetic dissection and more meaningful modelling of human disease and generation of humanised mice.

Mice will be used for this study since they are the least sentient species of mammal that have the following characteristics:

Individual mice within a given inbred strain or humanised mice generated from a single peripheral blood mononuclear cell (PBMC) or haematopoietic stem cell (HSC) donor are considered genetically 'identical', thereby reducing variability;

Mice reconstituted with human immune cells (humanised) and co-engrafted human cancers provide an excellent preclinical platform (the closest model system to a 'real human patients') where the complex cross-talk between the human immune cells and tumours can be studied in a niche-specific manner (ie, specific organs and tumour site).

Typically, what will be done to an animal used in your project?

This programme of work has 6 protocols that enable experiments to be conducted that meet our aims and objectives. The experiences of animals under these protocols will differ and below are detailed typical experiences for each:
Protocol 1: Engraftment of human cells (eg, PBMC, HSC) to generate humanised mice. Mice may be irradiated prior to engraftment of human cells (typically for neonates injected with HSCs). Mouse health will be then monitored post engraftment (eg, weight, posture) and blood sampling will be performed to assess the presence of human CD45+ cells in circulation, before being moved to any of Protocols 2, 3 or 4, for subsequent experimental procedures.

Protocols 2-3: Terminal bleeding for serum or cells. Mice (which may have been engrafted with tumours [Protocol 4]) will be administered a suitable non-recovery anaesthetic post therapy and bled by cardiac puncture to obtain blood for serum or cells.
Protocol 4: Immunotherapy in tumour-bearing humanised mice. In experiments designed principally to measure anti-tumour efficacy, reconstituted humanised mice will be injected with an appropriate tumour model and then administered treatments (such as a antibodies directly targeting cancer cells [direct targeting] and/or antibodies targeting the immune cells [immune modulatory antibodies] and/or other immune-modulatory substances) and tumour growth monitored over time through established means in experiments that may last several months, allowing us to determine if the treatments have cured the mice or delayed the tumour growth. Cured mice may be rechallenged with tumour or other sources of antigen to assess the induction of long-term immunological memory. A proportion of these mice may be monitored for their immune response during these experiments (peripheral blood or tissues).

Protocol 5: Immunotherapy in tumour-bearing immunocompromised mice. In experiments designed principally to measure anti-tumour efficacy, immunocompromised mice will be injected with an appropriate tumour model would be administered treatments (such as a direct targeting and/or immune modulatory antibodies and/or other immune-modulatory substances, eg, CAR T cells) and tumour growth monitored over time through established means in experiments that may last several months, allowing us to determine if the treatments have cured the mice or delayed the tumour growth. Cured mice may be rechallenged with tumour or other sources of antigen to assess the induction of long-term immunological memory (eg, CAR T cell persistence). A proportion of these mice may be monitored for their immune response during these experiments (peripheral blood or tissues).

Protocol 6: Tumour passage and establishing safe and humane tumour end-points. A minority of passaged (maintained) tumour models cannot be passaged in vitro whilst maintaining suitable characteristics for in vivo experimentation and therefore these models need to be passaged in vivo. Typically, mice will be administered a defined number of tumour cells and the tumours monitored and permitted to grow to the minimum point required to permit subsequent use of this tumour material for appropriately powered experiments. We constantly strive to use the most appropriate model to address our scientific objectives and therefore periodically need to acquire or develop new models which need to have their humane tumour end-points established to ensure we can reproducibly use the models for our objectives whilst minimising welfare impact.
When establishing a tumour for first time, typically, a small number of mice (which may have been given special diet to alter their body composition) would be administered tumour cells by an appropriate route and tumour growth and animal welfare monitored to determine a suitable end-point to ensure no unexpected or excessive harms are evident. These would be followed by pilot tissue sampling and harvesting or immunotherapy experiments to ensure the utility of the model.

What are the expected impacts and/or adverse effects for the animals during your project?

We typically identify mice by ear notching - this is expected to result in only mild and transient pain with no healing problems.

Some mice will be injected with various immune-modulatory substances using a combination of volumes, routes and frequencies. Injections will cause momentary needle stick pain, minimised by using the needle of the smallest suitable gauge. The minimum number of administrations and routes will always be used to achieve the scientific objectives. Typically, the animals will not receive more than 3-4 intraperitoneal injections a week or 2-3 intravenous injections a week. On some occasions they may receive 2 injections on a given day through a combination of different routes.
For some more invasive procedures, such as surgery, general anaesthesia will be used to ensure the animals feel no pain.

Mice, most of which are irradiated prior to engraftment of human cells, injected with human cells (either mature leukocytes or HSCs) are likely to experience moderate levels of severity. Presence of human cells may cause graft-versus-host disease (GvHD), where human immune cells attack mouse tissues.

GvHD may also occur in the humanised mice. The humane end-point for GvHD is based on Grade 2 disease symptoms (ie, 20% weight loss, hunching, moderate ruffling, denuded skin); generally hunching/impaired movement, low activity level and areas of denuded skin. If symptoms present then animals will be humanely killed by a Schedule 1 method. These symptoms may be exacerbated by immunomodulatory agents and immune cells. For some experiments, mice will receive tumour cells through various routes; eg, injected subcutaneously (under the skin) using a suitable vehicle or those spontaneously arising or induced. The tumours grow at their respective sites and can be measured over time using digital callipers, palpation or imaging. Mice are euthanised at a size that is deemed to not impact their normal behaviour. Throughout, the health status of the animal is the primary consideration used to define humane endpoints. Experiments will be terminated, or individual mice euthanised at the early signs of tumour-associated symptoms, such as weight loss up to, but not reaching, 20% body weight.

In many of our experiments, designed to measure immune changes, peripheral blood is taken from a superficial vessel. Pain from bleeding is controlled by suitable anaesthesia/analgesic with rapid healing and no long-lasting harm.

When the immune system is stimulated, for example with immunomodulatory agents, this can result in symptoms similar to those experienced during an infection (lethargy, fever etc). This can result in mice becoming less mobile, exhibiting pilo-erection etc. Typically, these symptoms are transient (first few hours) but can recur as the immune response develops (eg, after several days) potentially resulting in further effects such as weight loss. These effects are therefore carefully monitored with mice euthanised if their symptoms become more severe or pass defined humane endpoints.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Since all mice are immunocompromised and are to be engrafted with human cells, we expect moderate severity in all mice undergoing experimentation (100%).

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Whilst all attempts are made to reduce the use of animals by using in vitro methodology (in vitro experiments on human and mouse immune cells, explant studies and organotypic models), it is inevitable in work of this nature that in vivo investigation must be undertaken. The animal models detailed are critical to facilitate in vivo proof of concept and efficacy in an amenable and manipulable system, not possible in humans. We are investigating the in vivo effects and interactions between various arms of the immune system, with a view to therapeutic application in humans. These interactions occur between different tissues and organs, and as such they cannot be fully reproduced in vitro and so systemic in vivo studies remain fundamental to the study of complex tumour microenvironments, host factors and therapeutics. Further, advances, such as the availability of genetically altered mice and the development of more specific and sensitive techniques and reagents, continually permit a refinement and reduction in the types of experiments and the numbers of animals that are required (eg, as highlighted by the organisation 'Fund for the Replacement of Animals in Medical Experiments', http://www.frame.org.uk) and this is something to which I remain committed.

Which non-animal alternatives did you consider for use in this project?

In recent years, we have made large strides to make more use of human material at our institution. We now make extensive use of lymphocyte cones (blood cells collected from donors provided from NHS Blood Service) as a source of human immune cell populations for a range of immune assays and have built a range of clinical collaborations to allow us access to human tumour and healthy control material to study human cancer directly. Although powerful, these models are limited in both longevity and importantly complexity compared to in vivo systems and so we therefore still need to use appropriate mouse models to allow us to study our objectives in a whole-body system over a protracted time period and with facets such as biodistribution, pharmacokinetics and toxicity to be understood.

Further to this, and to try and better link between simple ex vivo culture assays and complex whole- body systems we have also embarked on a programme to develop appropriate human multi-cellular organotypic models (eg, human breast cancer, stroma and immune cells) for studying malignancy and the impact of body composition on inflammation and cancer.

It is difficult to quantify the reductions in mouse numbers that these changes have facilitated given that the experimental systems are complementary and not interchangeable as they address distinct scientific questions. However, without question, access to and greater use of human material to address basic scientific questions enables us to replace animals in some circumstances and this should continue to grow in the future.

Why were they not suitable?

We need to investigate the in vivo effects and interactions between various arms of the immune system, with a view to therapeutic application in humans. These interactions occur between different tissues and organs, and as such they cannot be fully reproduced in vitro. Each of the systems we have developed above, model different aspects of the immune response but none fully recapitulate the complexity and inter-relationships of a whole organism and so systemic in vivo models remain fundamental to our studies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

I have been working under current project licences held by other group leaders at our Centre and have been monitoring annual usage. The number above represents predicted numbers based on current activity and taking into account reductions due to our shift to using primary human tissues, where possible, as well as likely refinements over the coming years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our experimental design is always considered in light of how animal numbers can be reduced whilst ensuring meaningful and reproducible results. We are committed to reducing animal waste, implementing the three Rs, and maximising the reproducibility of research and so follow the PREPARE guidelines (https://norecopa.no/prepare) when considering our experiments.

As a first means to reduce the numbers of mice in our project, inbred mouse strains reconstituted with cells from a single donor (typically >20 mice can be engrafted with donor cells from a single individual) will be used to minimise variability of response and so allow reduced numbers to be used whilst delivering reproducible data. Related to this, we routinely use age- and sex-matched mice that have been bred in the same facility to minimise variability.

We have been using a range of tumour models for some time and so are well aware of the reproducibility of controls and appropriate mouse numbers required in tumour growth and therapy experiments. Where new models or treatments are introduced, we will first confirm expected humane endpoints and then perform pilot studies to inform the design of larger studies, including dosing regimens, as well as to monitor for any signs of adverse events. For example, 2 mice per group would be inoculated with a new tumour at different cell numbers to establish tumour growth kinetics etc., even where data exists in the literature, as we are cognisant of the impacts of the local environment, microbiome etc. on tumour growth and dissemination. Similarly, a single mouse would first be treated with a new

experimental treatment to judge safety, immune response etc, before proceeding to larger studies.

We are also cognisant of ensuring we minimise experimental bias. Accordingly, in experiments where we implement more subjective humane end points as part of a tumour therapy experiment (eg, abdominal palpation as opposed to calliper measurements), an experienced and treatment-group blinded animal technician is consulted regarding outcome.

Where we have useful data (from historical or pilot experiments), power analyses will be used to help guide the optimal numbers of mice needed for each experiment, taking into account expected magnitudes of impact. Power analysis is performed using the PS: Power and Sample Size Calculation programme:
https://biostat.app.vumc.org/wiki/Main/PowerSampleSize):

For immunotherapy experiments, where we use reconstituted mice, intra-group variability is reduced. From experience, we have found that we can use considerably fewer animals per group, usually 5, to detect such a difference at the 5% significance level in many of our models (e.g. MDA-MB-231 breast tumour cells).
For monitoring immunological responses we typically use 5 mice per group for each experiment. From our experience, this number of mice/group allows us to identify with a statistical power of 95% a 3-fold difference in the number of CD8 T cells between 2 groups at the 5% significance level.

Therefore, the proposed mechanistic studies will be performed with experiments utilising groups of 5 mice, in a standard vehicle versus drug manner. We aim to test the candidate antibodies and immune modulators (identified by prior human and murine in vitro experiments) for efficacy in a selection of tumours. Throughout, replicate experiments will be performed to ensure reproducibility and when appropriate studies may be combined to increase sample size. To assess animal survival, end-points will be plotted against time for each group in an experiment and the differences between groups will be analysed by appropriate statistical tests.

Should substances or tumour models be used for the first time, pilot studies will be performed on individual mice and humane endpoints established as indicated above. Substances will be administered and monitored after 1, 4 and then 24h for adverse effects before proceeding; a dose escalation schedule conforming to accepted practice will be used should adverse effects be predicted. When agents are used for the first time in the laboratory, their dose will be based on previous published data whenever possible.
When new tumour lines are introduced they have to go through quarantine to ensure safe introduction into the animal facility. As an additional measure of reduction, we use this period to also monitor growth and humane endpoints reducing the need for additional animal use. More recently, to reduce the number of mice used, we have started screening new tumour lines by PCR rather than engrafting them into quarantined mice.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Mice used across experiments are inbred and reconstituted with cells from a single donor at a time, thereby minimising intra-group variability and allowing reduced mouse numbers for experiments.

Experiments are always designed with the fewest animals consistent with obtaining statistically valid results. We have performed Power analysis and our own extensive experience with these models to determine the numbers of mice required to deliver statistically significant results. Where appropriate, small pilot experiments are carried out to determine factors such as dose or route of administration.
Where multiple inter-relating parameters are to be evaluated, to prevent use of excess mice, we will employ higher dimensional analysis tools. Significant technological advances have enabled more information to be obtained from one individual mouse than was previously possible (e.g. using multi- parameter flow cytometry and RNA-seq technology), enabling multiple parameters to be assessed simultaneously from small samples. These technologies thereby facilitate longitudinal studies and reduce the need to kill multiple mice at different time points to sample from the spleen for instance. For example, we have largely moved from 3 to 8 and 16 colour flow cytometry, reducing the sample input requirements accordingly.

During the course of our current PPL, we have instigated a policy that new in vivo experiments must be detailed on a ‘study-plan’ detailing: the aims, number and strain of mice, substances/cells administered, treatment dates, confirmation of required competencies and/or delegation of procedures, any known or anticipated adverse effects, and appropriate risk assessments. This has to be submitted to the PPLh and NACWO before an experiment is allowed to begin. This has facilitated a more detailed dialogue between PPLh and PILh prior to work and if necessary a discussion about the number of mice being used.

Other optimisations to reduce animal use include:
Tumour cells being stored frozen when possible to prevent mice being used to passage tumour in vivo.
Consideration of freezing additional animal tissues (whole and as single cell suspensions) when an animal is sacrificed to provide controls for staining in different immune situations, relieving the need to kill specific mice for this comparative purpose.
Similarly, harvesting tissues; blood (for serum production), spleens (for lymphocytes) and bone marrow (to generate macrophages) when appropriate mice are killed for other purposes.

The in vitro use of immune cells isolated from mouse tissues offers several benefits and aids in optimising number of animals used for in vivo experiments. Importantly, this strategy is in line with the 3Rs. Due to the relatively low numbers of cells required to perform in vitro assays, such experimental approaches allow the screening of a high number of drugs or combination thereof, with potential for in vivo activity, while minimising the number of animals required. Furthermore, they allow refining doses, and identifying potential mechanisms of action and adverse reactions, prior to in vivo testing. In addition, in vitro assays using mouse immune cells represent a powerful reductionist approach to study their activation. They allow the isolation and analysis of distinct cell subsets, and the direct assessment of drugs on each individual cell population, otherwise difficult to accomplish.

For monitoring immunological responses in vitro, we generally use 1 mouse per experimental setting with 3 technical replicates and repeat the experiment two to three times. From our experience, this number of mice allows us to identify with a statistical power of 95% a 1.5 fold difference in immunological responses (eg, cell proliferation,

upregulation of activation markers, cytokine expression, phagocytic uptake) between the control and treatment arms at the 5% significance level.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

When evaluating immune-modulatory and immunotherapy approaches, we begin with in vitro evaluation and progress to pre-clinical animal models when sufficient promise is obtained. Our in vitro studies may include assays to determine the ability of reagents to kill or affect the growth or survival of target cells, and to recruit immune effector mechanisms. Where appropriate, we will first establish effects on in vitro cell lines and ex vivo primary material. However, in vitro lines and primary cells become adapted to cell culture conditions and are not present in a complex multicellular tissue/organ (including immune cells) and therefore do not best recapitulate in vivo responses, hence the need for animal experiments.

In order to understand the mechanistic requirements for effective immunotherapy, cells may be obtained from genetically altered mice with deficient/altered immune effector systems. eg, control or reconstituted NSG and NSG-SGM3. For those reagents that show promise in vitro, we will determine if this translates into an in vivo effect using the most appropriate models. Typically, we would first examine reagents in the absence of tumour or other 'altered' host factors, to assess immune- modulation, without unnecessarily increasing the welfare impact to animals by adding to the cumulative harm from further interventions and/or activities of the tumour. We ensure humane end-points are established that minimise the harm to the animal without compromising the accuracy of the experimental data.

Why can’t you use animals that are less sentient?

Our work largely involves study of the immune system, which is a complex, multi-faceted, interacting system that is spread throughout the body and organ systems. The cellular and molecular interactions of the humanised mice are broadly similar to those of humans, allowing us to investigate clinically relevant immunotherapy strategies and their mechanisms in these animals - less sentient species have very different immune systems. More immature animals also do not display mature immune systems (the immune system becomes educated with age and development) that we seek to investigate and understand for clinical benefit. Anaesthesia reagents frequently interfere with multiple bodily functions which would alter the immune response and cannot be used for the extended periods required (days to weeks) to follow immune responses induced and so are not routinely used unless required.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We continually review our procedures and if unnecessary harms are identified or can be reduced, modified protocols are implemented through discussion with the NACWOs, PPL holder, academic lead for the animal facility and named vet as required. We also seek to ensure the best housing and conditions for the animals are provided.
Working under other PPLs within the Centre, we have instigated the changes below that demonstrate our commitment to refinement:

- We have moved to a new purpose-built animal facility that is more conducive to high quality in vivo research utilising a ‘barrier’ system meaning that all materials coming into the unit are clean and enter via a dedicated positive-pressure pass-through hatch. All personnel entering must now change into scrubs, and pass through an air-shower on entering (and leaving). The new facility also houses a separate quarantine facility that is no longer inside the main unit, but attached and accessible separately, with dedicated technicians only permitted.

- All new strains of mice entering quarantine undergo rederivation before offspring are allowed in the main unit. All new cell lines must be screened in mice in quarantine or by PCR, for the presence of pathogens before cells are allowed to enter the main unit. All mice in our new facility are housed in individually ventilated cages. These changes minimise the risk of infection with obvious potential implications for the welfare of the animals and the quality of the research. In addition, the IVCs permit less disturbance to the animals as an additional refinement.

- We have also implemented a number of new policies to improve welfare and reduce adverse experiences. All mice undergoing an injection now undergo a ‘second check’ by the person performing that procedure, within 30 mins - 4 hours. This was instigated to prevent animal welfare impact should an unanticipated adverse event occur. If a procedure is delegated to a technician, this second check also incorporates a ‘positive handover’ between the technician and the PILh to transfer the responsibility for the mice back to the PILh to ensure it is always clear where responsibility for welfare ultimately lies.

- In response to changing guidelines a number of further and specific refinements have been put in place, including:

- Mice are generally killed by dislocating the neck (Schedule 1), unless CO2 chamber is required.

- Mice are handled by ‘cupping’ or by gently moving them using a dedicated tunnel, prior to any restraint to ensure mice are calm and habituated to the person performing injections, serving to reduce anxiety when handling mice.

- Instigated a policy that needles are only to be used once. This is to prevent mice receiving an injection with a blunted needle. Initially during the early part of this PPL the refinement was for needles to be used less than 5 times, but latterly we implemented the new single use policy unless specific justification is provided that is approved by animal facility management committee.

- We have transitioned towards venesection as our primary means to take blood samples instead of tail lancing. While this is not always possible (for instance when mice are receiving concurrent intravenous injections) new PILh are now trained in venesection and established PILh who regularly bleed mice have undergone re-training.

- We also have a new system that records training competencies for individual PILh to ensure that training on all procedures is renewed every 3 years. Training is conducted by dedicated trainers using Detailed Operating Procedure (DOP) forms written specifically for this purpose. As PPLh I have access to these records, and reference is made to them on the 'study plan' (see above) ensuring that PILh also review their records regularly.

- We have regular animal facility users meetings (approx 3 times per year) to discuss issues that arise within the facility and these are well attended by the personal licence and project licence holders, named animal care and welfare officers, animal technicians, home office liaison and compliance officer, named veterinary surgeon and establishment licence holder. Any issues and incidents that PILh need to be aware of are discussed at this meeting. We have also embarked upon a strategy of ensuring that

- PPLh and PILh undergo regular refresher training at least every 5 years to ensure that they remain abreast of changes to best practices and aware of their responsibilities under ASPA.

- We have also adopted the practice of transferring male mouse nesting material, not substrate, to minimise male mouse aggression.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

There are a number of detailed publications and guidelines for the welfare and use of animals in cancer research that provide excellent guidance on the methodologies, study design and best practice that we will follow and adapt as appropriate to our research (Guidelines for the welfare and use of animals in cancer research: Workman, P., Aboagye, E., Balkwill, F. et al. Guidelines for the welfare and use of animals in cancer research. Br J Cancer 102, 1555–1577 (2010). https://doi.org/10.1038/sj.bjc.6605642).
We will continue to follow the ARRIVE guidelines https://arriveguidelines.org/ which provide a checklist of the minimum information required to be reported by groups using animals in research. ARRIVE guidelines are essential to help overcome issues in science such as reproducibility, reducing bias and the correct use of statistical methods of analysis. In addition, we will follow and consult NORECOPA https://norecopa.no/3r-guide : Norway's National Consensus Platform for the advancement of the 3Rs (Replacement, Reduction and Refinement associated with animal experiments) database platform and PREPARE https://norecopa.no/prepare (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines for better science experiments using animals to ensure that we are using the best models for our research.
Finally, regular communication within our research group and communication with our peers at seminars and conferences ensures we're aware of any new or updated best practice. Best practice information is also disseminated by our Named Information Officer.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have regular users meetings (approx 3 times per year) to discuss issues that arise within the animal facility and these are well attended by the personal licence (PILh) and project licence (PPLh) holders, named animal care and welfare officers, animal technicians, home office liaison and compliance officer, named veterinary surgeon and establishment licence holder. Any issues and incidents that PILh need to be aware of are discussed at this meeting. Further to this, this platform is used to disseminate information from ASRU, NC3Rs and other organisations and to provide details of training opportunities to enhance welfare and research practice. We have also embarked upon a strategy of ensuring that PPLh and PILh undergo regular refresher training at least every 5 years to ensure that they remain abreast of changes to best practices and aware of their responsibilities under ASPA. These advances are further communicated through our active team of NACWOs and regular email updates. We will also stay up to date with specific cancer groups, databases and alternatives (NC3Rs) for cancer models such as: https://resources.researchanimaltraining.com/faqs/breast-cancer-

research-alternatives-database; https://data.jrc.ec.europa.eu/dataset/352f7dfd-05cf-434b-a96a- 7e270dc76573.

44. Hepatitis E Virus (HEV) in Pigs: Understanding Infection Dynamics and Deciphering Prevention Strategies.

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Hepatitis E virus, pork food chain, prevention strategies

Animal types	Life stages
Pigs	juvenile, neonate, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To investigate and understand the dynamics of hepatitis E virus (HEV) in pigs, the mechanisms by which HEV may enter the pork food chain and options for mitigation thereof.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

There is epidemiological evidence which highlights the role of pork food products in human hepatitis E cases. To date, the mechanism with which hepatitis E virus (HEV) enters the pork food chain is unclear. The experiments proposed within this licence application will

investigate how hepatitis E virus might enter the pork food chain. In addition, it is intended to develop and test prevention strategies such as vaccines. Together, these data could be of use to inform farming practices and public health policies.

What outputs do you think you will see at the end of this project?

New information regarding the infection, transmission and prevention of HEV in pigs with associated publications. The production and trial of the first pig HEV vaccine for experimental use. Efforts will be made to maximise the use of any HEV positive material (e.g. faecal samples, tissues) derived from this project. Material remaining after analysis requirements for this project are complete will be catalogued and stored for use in future projects and if/where possible shared with colleagues working in the same field. This positive material may also be used as control material when setting up or updating diagnostic tests for HEV.

Who or what will benefit from these outputs, and how?

After the completion of each protocol, the information gained will be initially shared with colleagues/collaborators also working within the field of HEV research e.g., internationally via conference attendance or nationally within specific working groups such as the cross UK research HEV Working Group. Towards or at the end of the project, scientific publications are planned for each protocol. Once the project is complete it is anticipated that these data may be used to inform farming strategies to reduce transmission/infection of HEV, knowledge of how/why HEV enters the pork food chain. These data may also be used to inform governmental policy.

How will you look to maximise the outputs of this work?

It is planned to collaborate with academic partners for specific aspects of experimental sample analysis and also for the generation of products such as the HEV vaccine. Efforts will be made to publish all approaches, including those which may be unsuccessful thereby informing fellow researchers and minimising any duplication of efforts. Knowledge gained via this project will also be disseminated via national/international conferences and meetings. Knowledge which may relate to governmental policy or farming practices will also be shared with the appropriate stakeholders.

Species and numbers of animals expected to be used

- Pigs: 760

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

As the aim is to investigate the presence/transmission of HEV via the pork food chain, pigs will be used. Pigs aged between 6-10 weeks of age at the start will be examined in this programme. This age group was selected as our previous research indicates these age

groups are the most susceptible to HEV infection and therefore ideal for the proposed works. For the analysis of HEV on farms we will use all post-natal age groups of pigs to improve our understand the dynamics of HEV on farms.

Typically, what will be done to an animal used in your project?

In 5 protocols the pigs will be infected with HEV, orally in four protocols and intranasally in one protocol. Blood sampling after inoculation will only start after HEV has been detected in faecal samples. When blood sampling commences it will start every forth or fifth day and be no more frequently than every three days when a peak/high viraemia is observed (around 2 weeks after initial sampling is the earliest and reduced thereafter to once per week for up to 8 weeks maximum). Blood samples will be analysed in real time (i.e. not stored/batched until the end of the experiment). Faecal samples will be preferably collected from the ground. In one protocol only vaccines will be applied and blood samples be taken (approximately weekly for the duration of the experiment) and the Protocol for the on farm study only blood and faecal samples are to be taken (sampling will be occasional, i.e. every couple of weeks and most pigs will only be sampled once). At post-mortem, samples and tissues will be collected for analysis within this project and also catalogued and stored for future use/sharing with other research groups.

What are the expected impacts and/or adverse effects for the animals during your project?

All Protocols: HEV is not known to cause clinical signs in pigs and it is well documented that the virus is ubiquitous in the global pig population. The oral inoculation will be based on training pigs to voluntarily accept this. Sampling of faecal material should be unobtrusive (off the floor), but swabs may be required to monitor the status of individual animals. Blood sampling after inoculation will only start after HEV has been detected in faecal samples. When blood sampling commences it will start every fourth or fifth day and be no more frequently than every three days when a peak/high viraemia is observed (around 2 weeks after initial sampling the earliest) This is the point in time when pork products would contain the biggest risk for human health. Sampling would be reduced thereafter to once per week for up to 8 weeks maximum. Possible adverse effects with blood sampling include a slight risk of local haemorrhage occurring due to a tear/perforation of the veins. The risk of this is low as all blood samples will be taken by competent and experienced staff using appropriate equipment. The vaccines to be applied are using tested concepts and no other than local adverse side effects are expected.

To clarify that blood sampling would only occur at three days at high levels of viaemia, ie to identify such as best as possible as it is considered the period where pork producers would contain the biggest risk for human health. We have previously, in similar experiments, not observed aversity of the animals.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild, 100% of pigs. HEV infection does not cause any clinical disease in pigs, the vaccines to be applied are using tested concepts and blood sampling is only expected to cause

temporary pain or distress, accordingly the severity rating is mild.

What will happen to animals used in this project?

- Killed
- Kept alive at a licensed establishment for non-regulated purposes or possible reuse
- Set free

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In order to investigate how HEV enters the pork food chain, infection/transmission of HEV in pigs and prevention strategies such as vaccines and associated immune responses, there are currently no alternatives (i.e., no *in silico*, *in vitro* or *ex vivo* techniques) available to replace the use of pigs.

Which non-animal alternatives did you consider for use in this project?

A literature search (open and specifically using NCBI) was done for potential non-animal alternatives to the work described here. However what was found did not provide the whole animal response required to understand the infection/transmission dynamics and then the impact of any subsequently prevention strategies. There is insufficient information out there to use for modelling as well, hence the requirement for this work. We are separately also already using both single and complex cell culture approaches, including liver organ models, but the questions raised here cannot be addressed through such so far.

Why were they not suitable?

None of the non-animal alternatives is able to deliver the complexities of multi-organ interactions we are looking to study here relating to the pathogenesis of disease. Such whole animal response are required to understand the infection/transmission dynamics and then the impact of any subsequently prevention strategies. There is insufficient information out there to use for modelling as well, hence the requirement for this work.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The project licence has 5 objectives:

1) The impact of HEV Genetic Diversity on Transmission
2) Alternative pathways of HEV infection
3) Impact of Age on Susceptibility to HEV infection
4) Investigating a HEV vaccine for pigs
5) Investigating HEV on farm

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To minimise variation standard protocols will be applied for example for preparation challenge virus and for performing the inoculations and laboratory analysis. To control variability and allow smaller group sizes animals will be of similar age, weight, with a good health status.

Our experimental design will be based on group sizes that should show significant differences so that experiments will not need to be repeated because results were inconclusive. Our previous work has provided us with a good estimate of the likely variation to be expected so that a power analysis can be used to estimate the correct number of animals more accurately, which could otherwise be over- estimated. Statistical input for studies will be sought to ensure validity of data from the organisations bio-statisticians as well as using the NC3R’s experimental design assistant.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Efforts will be made to maximise the use of any HEV positive material (e.g., faecal samples, tissues) derived from this project. Material remaining after analysis requirements for this project are complete will be catalogued and stored for use in future projects and if/where possible shared with colleagues working in the same field. This positive material may also be used as control material when setting up or updating diagnostic tests for HEV.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project will use pigs to investigate hepatitis E virus (HEV) infection and transmission. Pigs will be given acclimatization time to become familiar with their surroundings, their pen mates and staff who will be responsible for their daily care. Where applicable, the acclimatization period allows the pigs to become familiar with the equipment that may be used i.e. without actually conducting procedures.

As HEV does not cause clinical symptoms in pigs, suffering due to infection will be minimal. HEV will be inoculated orally for the majority of protocols, and intranasally in one protocol, both of these procedures should not cause harm or pain. To avoid distress the pigs will be trained to allow the inoculation to take place for a treat.

Blood sampling will be conducted for each protocols, this will be eased by the use of positive enforcement training. This reduces the stress involved to the animal by training the animal to cooperate with the procedure and by conducting it in a quiet environment. Pigs are intelligent animals and will remember receiving a reward (e.g. food treat) after the procedure, which increases their cooperation on subsequent occasions.

Laboratory analysis of key samples will be carried out whilst the experiment is in progress to inform and adjust the blood sampling frequency, the intention of minimising sampling necessary to achieve the experiment’s aims as well as the impact on the welfare of the animals.

Faecal samples will also be collected throughout the protocols, these samples will be collected from the ground and so non-obtrusive.

Why can’t you use animals that are less sentient?

HEV genotype 3 mainly infects pigs (incl. wild boar) and humans, while rodent species are not susceptible to these and have a significantly different biology. Therefore in order to investigate HEV transmission from pork meat to the food chain no alternative than the use of pigs has been identified.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Feedback will be taken from staff following their daily observations e.g. in case of any unexpected health and welfare issues. The staff involved with the implementation of each experiment will be fully and formally briefed before the start of each of the studies (pre-start meetings).

Blood sampling is stressful for pigs, to reduce the stress involved the pigs will be trained to cooperate with the procedure and by conducting it in a quiet environment. Training involves receiving a reward (e.g. food treat) after the procedure, and it increases their cooperation on subsequent occasions.

The pigs will be observed closely following blood sampling and in the unlikely event of any animal showing signs associated with stress of haemorrhage, e.g. pallor, shaking, depression, shivering, will be treated immediately by the Named Veterinary Surgeon.

The protocol studying HEV infections on farm at POLE, the animals will be retained in their social groups and re-homed into their farm after use.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

NC3Rs:

Training animals: https://nc3rs.org.uk/3rs-resources/training-animals

Blood sampling - Pigs: https://www.nc3rs.org.uk/3rs-resources/blood-sampling/blood-sampling-pig.

Humane endpoints: https://nc3rs.org.uk/3rs-resources/humane-endpoints

Euthanasia: https://nc3rs.org.uk/3rs-resources/euthanasia

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly review information from the NC3Rs web site and newsletters as well as advances in procedures resulting in 3Rs such as from colleagues in the organisation or from relevant external publications which are regularly disseminated across the organisation via an internal newsletter. As an experienced laboratory we will be in regular contact with other researchers world-wide and new knowledge on procedures of similar studies will be taken into account. Relevant advances will be incorporated into study designs and procedures.

45. Investigating the neural basis of episodic and spatial memory

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Memory, Alzheimer's, Episodic, Hippocampus, Entorhinal Cortex

Animal types	Life stages
Mice	adult, aged, juvenile
Rats	juvenile, adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To determine the neural systems and mechanisms that support episodic and spatial memory in health and disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Understanding episodic and spatial memory is important for both healthy living and for certain clinical conditions. These memory processes enable us to carry out everyday tasks safely and efficiently (remembering to turn off the oven and where the car is parked) but also help shape our personalities and values. Breakdown of these processes are some of the earliest and most debilitating symptoms of dementia, particularly Alzheimer's disease

(AD), but even healthy ageing is associated with decline of these memory systems. Understanding how the brain facilitates these memory processes enables us to not only help identify and test treatments for dementia but can also help us identify lifestyle factors that will minimise memory decline in normal healthy ageing.

The use of animals is critical to the project success. This enables us to examine the system at molecular and genetic levels and also to manipulate the system to examine which are the critical circuit mechanisms that support memory. These studies are not possible in humans.

What outputs do you think you will see at the end of this project?

This work is expected to produce outputs that further our understanding of the neural and psychological mechanisms underlying spatial and episodic memory. It will advance our understanding of the neural systems underlying these cognitive functions in both healthy brains and diseases which result in memory impairment. The objectives are stated below, and the specific outputs are listed by objective.

1. What are the medial temporal lobe network mechanisms that underlie episodic and spatial memory?

We will produce data that will further our understanding of the neural mechanisms underlying episodic and spatial memory. Specifically, we will test the hypothesis that there is functional dissociation within the hippocampal-entorhinal network and that different components of episodic memory (spatial, temporal and contextual) are processed and integrated by different network components. We will also examine different properties of the system that might contribute to memory such as neurogenesis and place cell remapping. We will produce many types of data (e.g. behavioural, anatomical, electrophysiological) that will refine our understanding of how we remember our conscious experience. The main outputs will be academic papers, but we will also publicise the findings through talks and presentations at both scientific conferences and public engagement events. All published data will be deposited in public repositories for other researchers to use.

2. What are the functional roles of the genes and hormones associated with Alzheimer's disease and other dementias in episodic and spatial memory?

We will produce data examining the roles of hormones and genes associated with AD and other dementias. Specifically, we will test whether these hormonal and genetic targets affect memory mechanisms within the hippocampal-entorhinal network and potentially go onto examine whether they prevent memory impairments in models of AD and other dementias. Again, the main output will be academic papers but we will also publicise the findings through talks and presentations at both scientific conferences and public engagement events. All published data will be deposited in public repositories for other researchers to use.

Who or what will benefit from these outputs, and how?

Who will use these data?
The data will be of benefit to the research group and establishment to push scientific boundaries. The data will also be an invaluable resource for systems and computational

neuroscientists who wish to model the function of the brain and specifically memory systems within the brain. By increasing the validity of these models, it is predicted that they can be used to address questions of how memory is processed in the brain and may ultimately lead to a reduction in the number of animals needed for future research. It will also be useful to applied researchers aiming to understand the mechanisms that are affected in the very early stages of AD and other dementias. While it is not possible to estimate how many people will benefit from these findings these data will be of use both to basic scientists examining the early degenerative processes in AD and also to applied researchers within industry interested in developing therapeutic strategies for AD. Given the prevalence of AD and the ageing population this will of wide interest.

How will you look to maximise the outputs of this work?

The lab group collaborates widely with researchers within and beyond the field. This promotes best practise sharing and exchange of ideas within the field and also helps to situate the research within wider theoretical landscapes. This is important as modern neuroscience techniques allow the examination of questions at very precise levels of detail which can result in researchers failing to take into account the bigger picture of how the research fits into the field and ultimately influences society. Examples of how the lab does this at different levels include:

University level: we collaborate widely with other groups in the university including interdisciplinary networks.

Field level: we attend specialist memory conferences such as the Spring Hippocampal Research Conference and also larger general neuroscience conferences at both national (British Neuroscience Association) and international (Society for Neuroscience) levels to disseminate findings and develop new collaborations.

Interdisciplinary level: we collaborate with a range of researchers outside of neuroscience including scholars in Biology, Psychology, Anthropology, Philosophy and English literature. This helps situate the research in a wider context and prevents us from becoming too narrowly focussed.

Public engagement level: the lab is very active in disseminating findings to the public. We do this through popular public engagement programmes such as Cafe Scientifique and Pint of Science as well as in collaboration with charities such Alzheimer's Research UK.

Finally, we are committed to open science. We publish in open access journals and also make our data freely available.

Species and numbers of animals expected to be used

- Mice: 4000
- Rats: 1250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use adolescent and adult rats and mice. Rodents brain structure is similar enough to humans to allow a homologous approach that allows us to identify equivalent neural structures and networks in rodents and humans. Our research centres on the hippocampus and surrounding medial temporal lobe. The hippocampus in particular is very well preserved across mammalian species meaning we can have reasonable confidence that it serves similar functions in rodents and humans. Rodents are also capable of remarkable memory performance which allows us to use behavioural tasks that test the human memory processes upon which this project focusses. Certain studies may examine older animals as memory declines with age and one of our research interests is how neural mechanisms within the hippocampus (and associated regions) change in older animals.

Typically, what will be done to an animal used in your project?

Typical experiments will aim to monitor and / or manipulate brain function as rodents are carrying out behavioural tasks that test memory. These studies involve 3 stages that may be used individually or in combination:

1. Behavioural tests will usually involve testing rodents over a number of weeks to gain sufficient statistical power to allow meaningful conclusions to be drawn. Rodents would typically be tested once a day on either spontaneous behaviour (e.g. exploring objects in an arena) or trained behaviour (e.g. learning to dig in a bowl to gain a reward). This may involve restricting diet before testing to ensure that rodents are sufficiently motivated to perform a task for food treats. Animals are then fed a normal amount of food after testing and weights are monitored daily to ensure healthy weight gain. Behavioural testing by itself would be subthreshold.

2. Monitoring brain function can either be done in awake behaving animals or by examining brains after death. To monitor brain activity as rats are carrying out memory tests we would measure electrical signals in the brain. Animals would undergo surgery to implant permanently indwelling electrodes or probes that allow us to monitor the activity of individual brain cells and correlate this with memory performance. Animals would undergo one surgery and following recovery would be tested once a day for a number of weeks. Anaesthetics (general and local) are administered pre- and during surgery, and painkillers are administered the day before, immediately prior to, and two days after surgery to reduce post-operative pain. Procedures involving surgery are up to moderate severity level. Alternatively in different animals, we can monitor brain function after death. By using labelling of recently active cells we can measure which brain structures and networks were active as rats carried out a memory test. This labelling may involve the surgical injection of a marker into the brain to label specific chemicals or genes. Animals will only ever have one surgery.

3. Manipulating brain function allows us to understand whether specific brain regions or mechanisms are critical for memory. For example, we might use genetic techniques or drugs to increase or reduce activity in a particular region and measure the effect on memory. This would typically be done in one of two ways. The first method would be the use of genetically modified mice that will have genes permanently inserted to or

deleted from their genome that modify brain function. Alternatively, this would involve surgically injecting a drug or genetic agent that would alter the activity (increase or reduce) of a particular brain region. In both cases we can then test whether that brain region or function manipulated is critical for memory by examining memory performance relative to control animals with normal brain function. Again, surgical procedures would be of moderate severity. Genetically modified mice would have minor changes in memory function but no other overt changes to behaviour and so would be mild severity.

Behavioural experiments would normally last for 4-16 weeks. It may be interesting to examine memory processes in ageing and in that case animals might be kept until they are up to 24 months of age with testing taking place between 15-24 months depending on the question. Again, duration of testing would normally be 4-16 weeks.

What are the expected impacts and/or adverse effects for the animals during your project?

We would not expect any impact or adverse effects from behavioural testing in isolation. Our food restriction protocol is only designed to motivate animals to perform tasks for treats with a normal daily quantity of food being given each day after testing. Any changes in behaviour would be reported to the facility's NACWO and NVS for advice and discussion.

Genetically modified animals will show only minor changes to memory and no physical changes. These changes will only be detectable with memory testing and their everyday behaviour and appearance should be indistinguishable from normal animals.

Animals that have had surgery, either for the surgical injection of a drug or genetic agent or for implantation of electrodes would be expected to experience transient moderate levels of pain but will recover quickly and be normal within 48 hours of surgery. Animals with implants will take some time to acclimatise to the headcap that secures the implants on the skull. This happens quickly though and we would expect them to be acting normally within 48 hours with only occasional scratching as part of normal grooming. As with humans these animals would be given anaesthesia and pain killers to minimise discomfort and pain. Some weight loss immediately after surgery would be expected but this should be recovered within a few days and then normal weight gain would be expected.

We may examine memory in older animals. These animals will inevitably develop mild age-related decline in general health. Once animals reach 15 months of age, they will be monitored weekly for general health using detailed checklist developed by animal welfare researchers. Any animals showing health changes that might be expected to escalate beyond a mild severity limit would be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Experimental Mice (protocol 1): 10% subthreshold, 40% mild, 50% moderate

Experimental Rats (protocol 1): 20% mild, 80% moderate

Breeding and maintenance of GA Mice (protocol 2): 90% sub-threshold, 10% mild.

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The proposed experiments aim to examine the neural mechanisms underlying learning and memory. This involves studying an organism that can demonstrate that it has learnt information and that it can remember that information for long periods of time. The available options here are humans and animals. Human subjects are used whenever possible to better define memory processes, however in order to examine how the brain processes memory we need to carry out experiments that monitor brain function or manipulate the system in some way (through genetic manipulation or drugs). Monitoring brain function can be done in humans using methods like fMRI. However, these methods have poor temporal and spatial resolution. In order to gain a real understanding of how the brain performs psychological functions we must examine the activity of individual neurons. This is not possible in human subjects.

Which non-animal alternatives did you consider for use in this project?

At the beginning of each project we carry out literature reviews to examine possible alternatives for the use of animals in our experiments. These involve searching scientific databases such as web of science and pubmed and also specific 3Rs resources such as the NC3Rs website and Norecopa. We use search terms such as 'in silica' and 'computer-aided' combined with project specific terms such as memory to search for both scientific papers and websites that might offer suitable alternatives for the use of animals. Currently the only possible alternatives are cell culture, organoids and computational models/neural networks.

Why were they not suitable?

Other alternatives include cell cultures which are clearly unsuitable for examining psychological processes as single cells cannot produce complex cognitive functions. Organoids are also of limited value as we are interested in specific systems and networks within mammalian brains which these would be unable to model. Finally there are computational models of neural networks. Models can be very useful for generating hypotheses and where possible these are used. However current models are necessarily simplistic and so cannot simulate many memory processes.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Advice on proposed experiments and methods of analysis of the results will be taken from local statistical experts. Where relevant, we will test multiple factors in the same experiment rather than the one-thing-at-a-time approach, to maximise the information obtained from the minimum number of animals. Sample sizes for our experiments are estimated from past experiments where possible. Otherwise, we will use previous experiments (ours, or from the literature) to select sample sizes. Different techniques and behavioural tasks produce different patterns of data so group sizes will vary to ensure meaningful results but on average most studies will use 10-12 animals per group for rats and 12-14 for mice as mice typically show slighter poorer memory than rats.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will use best practise to monitor new ways of measuring memory in the literature that reduce variance and increase power. As an example, we were recently part of a group of universities awarded an NC3Rs grant to assess a new apparatus that allows multiple object recognition trials to be run for each animal on a given day. This increases the data we can obtain from one animal and decreases overall group size.

We also monitor the literature to ensure we are using the most powerful tools available to us. For example, we continually monitor advances in electrophysiology that allow us to record as many cells as possible from each animal. Modern silicon probes should allow us to record from 100s of cells in each animal which is a large increase on traditional metal electrodes. This will reduce the number of animals needed.

We will also take statistical advice from local experts and tools such as the NC3Rs' experimental design guidance and experimental design assistant (EDA) to ensure that randomisation and blinding are carried out correctly.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

To ensure efficient mouse breeding programmes we use colony management software that also allows us to monitor numbers of mice over time and plan efficient breeding; reducing numbers between behavioural experiments and ramping up at the right time to ensure sufficient numbers for experiment.

Pilot studies will be performed where appropriate to test new methods and behavioural tests of memory. These will also give us the data we need to make calculations of group sizes for future studies.

We also make the most use of each animal as possible by freezing brain tissue at the end of experiments. This might involve keeping brain sections from animals that have

performed behavioural tests of memory. This is a valuable resource for us and other researchers who can make use of the tissue to test new hypotheses about how the brain processes memory that arise with new developments in the literature.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The species used in the current application are rats and mice. These are used for a number of reasons. We require a species that shows the same type of memory process as humans and has a brain similar in structure. This ensures that findings from these studies can be used to make conclusions about memory processes in humans. This rules out fish, insects and birds. The rodent brain is highly analogous to the human brain with the hippocampus being very well preserved across species. Rodents have also been shown to have largely similar memory processes making them a good model for the current set of studies. We need to use adult rodents as juvenile rodents do not show the same type of memory processes. For these reasons adult rats and mice are the most appropriate species for this application.

We will make use of procedures with the lowest levels of pain and suffering for the animals. We will consult with the vet to ensure that we are using the best possible anaesthesia and painkillers for surgery. We will also keep up to date with current methods for recording and manipulating the brain and adopt new methods that will result in lower levels of pain and distress wherever possible.

As an example of this over the last 10 years we have transitioned from creating permanent lesions within the memory network to using genetic methods to manipulate the network. While these still require surgery they do not result in neurons being destroyed and so have fewer long term impacts on the welfare of the animals.

When using genetically modified mice we will not be developing new strains. We will use mice that allow us to manipulate the memory system. These mice will show no physical effects and only minor memory issues. We may also use mice that are genetically altered to mimic AD such as the 5xFAD mouse. In these cases we will source the model with the least adverse effects that allow us to address our scientific question. This will be done by consulting the literature, the NACWO, the NIO and the vet.

Why can’t you use animals that are less sentient?

The aim of our studies is to understand how the brain supports human memory. To do this we need an animal that has memory processes similar to our own and similar enough brains to make comparison across species meaningful. Rodents have a memory system in

the brain which is very similar to humans and have also been shown to have sophisticated memory performance making them an ideal choice for these studies. Younger animals do not have well developed memory and the need to observe conscious behaviour (to infer memory) rules out terminally anesthesised animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Rats are socially housed at all times. Indeed one aspect of memory we are studying is environmental enrichment where animals are kept in large cages with numerous toys, mazes. They may also contain a running wheel and house larger group sizes to allow for more complex social interactions. We recently adapted our cages to ensure that animals with implanted electrodes can be housed socially and with enrichment rather than being single housed. This work was presented at an NC3Rs meeting for researchers and facility managers and technicians.

Where surgery is necessary pain killers are given before, during and after surgery. Soft bedding, wet mash and supplementary heat will be provided to help mitigate the pain.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Where surgery is necessary this will be done aseptically based on local operating guidelines which are based on the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery and have been approved by the local Ethics Committee. We will employ the PREPARE guidelines and the Guiding Principles for Behavioural Laboratory Animal Science (LASA, BAP, BNA, and ESSWAP) when planning and conducting our studies. In addition, we will use the 3Rs resource library for husbandry and in vivo techniques (e.g. Grimace Scales to assess pain).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will engage in regular communication with the highly skilled technical team at our facility, to ensure we stay up to date on techniques to ensure the best possible welfare outcomes arising from our research methods. We receive regular updates from our Home Office Liaison Officer about NC3Rs initiatives and workshops which we will attend when relevant. We also regularly check the NC3Rs website. We will also perform literature reviews for new methods at the beginning of each project (roughly annually). We will also attend international conferences which include trade fairs to keep up to date with the cutting edge techniques and equipment in the field.tives and workshops which we will attend when relevant. We also regularly check the NC3Rs website.

46. Breeding, maintenance, assisted reproductive techniques and imaging-based characterisation of Genetically Altered (GA) animals for use in regulated procedures

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Genetically altered (GA), Cryopreservation, Breeding and maintenance, Superovulation, Rodent

Animal types	Life stages
Mice	adult, juvenile, embryo, neonate, pregnant
Rats	adult, juvenile, embryo, neonate, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Breeding, maintenance, assisted reproductive techniques and imaging-based characterisation of Genetically Altered (GA) animals for use in regulated procedures for the duration of 5 years. It will facilitate the efficient and ethical management of each live resource throughout the duration of various research programmes.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could

be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

It is important to undertake this work because it will allow the creation of new genetically altered (animals have had deliberate changes made to their DNA, for example by adding or removing genes, to give them a specific characteristic) rodent lines. It will allow for the production of mice and rats with a known health status. It will allow for the cryopreservation or recovery of genetically altered (GA) rodent lines according to research needs on other project licences by preserving animal models of critical scientific importance without the need to maintain active breeding colonies etc. It will ensure that GA lines can be maintained and it can allow for the characterisation of phenotypes by imaging. The centralisation will allow for all activities to be performed and maintained in one place to support researchers and third parties to produce GA animals.

What outputs do you think you will see at the end of this project?

The overall objective of this project licence is to support researchers and third parties to produce new GA animals of known health status that are required for research whilst minimising animal usage through effective cryopreservation and recovery of lines. Diagnostic processes may be used to investigate anatomical and functional phenotype (observable physical properties of an organism) characteristics. Alternatively, suitable GA animals may be transferred to other project licences for experimental purposes.

Who or what will benefit from these outputs, and how?

Research groups from Biotechnology companies or Academic institutes will gain access to relevant models to fulfil research programme requirements. Direct contact with these research beneficiaries will allow for the continuation of downstream knowledge and constant evolvement to fulfil scientific requests.

How will you look to maximise the outputs of this work?

To maximise the outputs of this work, techniques will be continuously refined and the most appropriate methods adopted. Breeding will be continuously reviewed to ensure efficiency and to minimise animal wastage. Only viable groups will be used to minimise repetition of work and to ensure high standards. Sharing of information and knowledge across other facilities will prevent unnecessary breeding and/or experiments being performed. Publications of both successful and unsuccessful research will be promoted.

Species and numbers of animals expected to be used

- Mice: 11150
- Rats: 3840

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice and rats are the least sentient species suitable for the third party research that the programme of work is expected to support. Animals of reproductive age will be used for breeding purposes. Scientific data generated from well established disease models in rodents are translatable to that seen in human health.

Manipulative genetic technologies have been developed in the mouse for over 30 years to an extraordinary degree of sophistication. The unique genetic tractability of the mouse is based on the Nobel Prize winning ES cell technology but is also supported by detailed knowledge of the mouse genome which provides critical annotation as well as access to genomic resources.

Typically, what will be done to an animal used in your project?

Superovulation (Drug-induced release of multiple eggs for use in assisted reproductive technologies such as in vitro fertilisation (IVF) - synchronise the oestrus cycle (the cycle of reproductive activity shown by most sexually mature nonpregnant female mammals) and stimulate the release of a larger than normal number of embryos which can be harvested between 0.5dpc (days post coitum (sexual intercourse)) and 3.5dpc following a Schedule 1 method of humane killing. The resulting preimplantation embryos can be used for the generation of new transgenic mouse and rat lines via microinjection or electroporation, used as donor oocytes (immature egg) in an IVF or used directly for superovulation.

Embryo transfer - Surgical or NSET (non surgical embryo transfer) / TCET (transcervical embryo transfer) preimplantation embryos into the oviduct or uterus of females rendered pseudopregnant (the development of signs of pregnancy without the presence of an implanted embryo or foetus) by vasectomised males. This is necessary for the recovery of all lines being generated or recovered.

Vasecetomy (surgical procedure that results in male sterilisation)- Generation of sterile males to render oestrus selected females pseudopregant, which will subsequently be used as embryo transfer recipients. The most refined surgical procedures will be followed e.g. scrotal sac vasectomy instead of the abdominal route. Genetically sterile males will be used whenever possible.

Breeding and maintenance - Genetically altered (GA) mouse and rat colonies will be established or maintained on this protocol either as a result of being recovered by embryo transfer or being purchased from an approved supplier to send and distribute within the establishment for research purposes. The most efficient colony management techniques will be used and accurate breeding and welfare records will be maintained to monitor the condition of the colony.

Generation of founders - this protocol involves the genetic manipulation of eggs / blastocytes (a structure formed in the early embryonic development of mammals) / embryos (is the initial stage of development for a multicellular organism) for the production of novel genetically altered mouse and rat lines.
Cryopreservation (preservation of cells by cooling them below the freezing point of water) and assisted reproductive techniques - Archiving unique mouse and rat lines will be predominately by cryopreserving spermatozoa (is a motile sperm cell, or moving form of

the haploid cell that is the male gamete) from one or more males that have been identified as carrying the mutant allelles of interest. Animals will be humanely killed and the sperm harvested post mortem. In vitro fertilisation (IVF) sessions will be performed to confirm the viability of cryopreserved material and to help optimise future recovery attempts. This will require the superovulation of suitable donor females to accomplish this. Typically embryo culture will confirm viability of generated embryos however on occasion it may be necessary to transfer embryos into pseudo pregnant recipient females.

Experienced technicians may generate embryos for cryopreservation through superovulation and natural mating's or through IVF, harvesting donor females between 0.5dpc and 3.5dpc. Harvest details are recorded to monitor and optimise the productivity of each mouse line and against each genetic background. Embryo thaw and culture protocols can be used to infer viability, however it may be necessary to transfer embryos into pseudo pregnant recipient females.

Imaging - On occasion GA animals bred under this licence will have imaging performed to assess developmental disorders associated with the eye. Using historical data <2% of animals generated on this licence will be expected to exhibit non harmful eye related phenotypes.

What are the expected impacts and/or adverse effects for the animals during your project?

GA animals used in this licence are not expected to exhibit any harmful phenotype but if they do they will be humanely killed immediately.

Animals that have undergone surgical implantation of embryos may have some temporary discomfort following surgery. Although its expected to be seen infrequently, if wound breakdown or infection does occur the animal will be humanely killed immediately.

Animals that have undergone vasectomy may develop wound breakdown of infection and will be humanely killed immediately.

Females will be of an appropriate size, if they are to be mated. Over rigorous males will be replaced.

Any animal showing any deviation from normal health or well-being will be will be humanely killed immediately.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Superovulation - Mice 100% mild. Rats 100% mild

Embryo recipients - Mice <20% moderate, mild 80%. Rats <20% moderate, mild 80%

Vasectomy- Mice 100% moderate. Rats 100% moderate

Breeding and maintenance - Mice 100% mild. Rats 100% mild

Generation of founders - Mice 100% mild. Rats 100% mild

Imaging - Mice 100% mild. Rats 100% mild

What will happen to animals used in this project?

- Killed
- Used in other projects
- Kept alive at a licensed establishment for non-regulated purposes or possible reuse

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Rodents are commonly used in scientific research for several reasons. First, their genetic similarity to humans allows us to study biological processes that are comparable to those in humans. Additionally, mice and rats are easy to maintain, have a short generation time, and can be produced in large numbers. These factors make them valuable models for investigating various aspects of human health and disease. Researchers can manipulate mouse and rat DNA to study specific genes and understand both human physiology and the causes of diseases.

Which non-animal alternatives did you consider for use in this project?

Due to the nature of the work being performed, we are unable to use or develop non-animal alternatives.

Why were they not suitable?

There isn't currently any non-animal models that accurately demonstrate this type of work.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the number of animals we will use based on client demand and our experience of previous colony management.

What steps did you take during the experimental design phase to reduce the number

of animals being used in this project?

Historical data will be used to ensure all breeding plans minimise the number of animals used whilst maximising the number of animals generated, minimising the chance of study repetition.

Where possible, we will consult with clients prior to the commandments of work to determine the most appropriate model.

We will consult "The Home Office efficient breeding tool for GA breeding assessment" to ensure that we are keeping the number of animals produced to a statistical minimum.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

A colony management system will be used to monitor the number of animals used for each procedure. Breeding plans will be closely monitored and adjusted based on actual production rather than on predicted numbers. We will request information from clients to set appropriate framework(s).

Tissues from surplus animals will be cryopreserved and shared amongst the research community and third parties.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.
During this project we will be using superovulation, embryo transfer, vasectomy, GA breeding without harmful mutations and imaging.

The doses and routes used are well validated and are not anticipated to produce any adverse effects, beyond transient discomfort due to the injections.

Embryo transfer - Animals will be humanely killed 48 hours post their expected delivery date.

Genetically altered animals used in this licence are not expected to exhibit any harmful phenotype but if they do they will be immediately humanely killed.

Animals that have undergone surgical implantation of embryos may have some temporary discomfort following surgery. The advice from The Named Veterinary Surgeon (NVS) will be consulted if required to discuss pain management. If wound breakdown or infection occurs the animal will be immediately humanely killed.

Animals that have undergone vasectomy may develop wound breakdown or infection, if wound breakdown or infection occurs the animal will be immediately humanely killed.

Singularly housed animals will be avoided as much as possible.

Females will be of an appropriate size, if they are to be mated. Over rigorous males will be replaced.

Bedding and nesting material, refuges, and gnawing sticks are the most commonly used enrichment resources for mice and rats and will be provided in every cage.

Why can’t you use animals that are less sentient?

Rodents are the least sentient species that we can use to complete the objective of this licence. They have genetic similarity to humans allowing us to study biological processes that are comparable to those in humans. Additionally, mice and rats are easy to maintain, have a short generation time, and can be produced in large numbers. These factors make them valuable models for investigating various aspects of human health and disease. Researchers can manipulate mouse and rat DNA to study specific genes and understand both human physiology and the causes of diseases.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will use applied techniques and standardised protocols issued by the Home Office.

We are driven to constantly evolve and make data driven improvements to our methodologies.

Where possible, singularly housed animals will be avoided. Throughout the duration of the protocol the animals will have continuous access to food, water and suitable enrichment.

Animals will be immediately monitored post injection and checked again at the end of the day. The animals will be checked every day where / if necessary and the frequency of the monitoring may be increased should it be required. If problems arise we can act quickly to prevent further problems. The Named Veterinary Surgeon (NVS) will be consulted if required to discuss pain management.

If complications arise during the imaging procedure the animal will be immediately humanely killed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Efficient Breeding of Genetically Altered Animals Assessment Framework

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Attendance at relevant conferences and subscription to relevant newsletters and/or documents. We will exchange information through AWERB, our other facilities, our in-

house compliance team and third parties.

47. Characterising and Inhibiting Vascular Disturbances

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Myocardial infarction, Microvessels, Ageing, Diabetes, Chronic Kidney Disease

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to understand what problems occur in the smallest blood vessels of the heart after a myocardial infarction (MI), commonly called a heart attack. We will investigate how three common co- morbidities, namely increasing age, diabetes and chronic kidney disease, affect blood vessels of the heart and whether they increase the susceptibility of these tiny blood vessels to greater damage after an MI.

Importantly, we aim to develop novel strategies that can specifically inhibit disturbances within the blood vessels of the heart and ensure these therapies remain effective in the setting of co- morbidities.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cardiovascular diseases are the number one cause of death worldwide. Most of these deaths are due to a myocardial infarction (MI), commonly called a heart attack.

An MI occurs when one or more of the large blood vessels (arteries) of the heart become blocked which subsequently deprives the heart muscle of essential oxygen and nutrients. For many patients, this blockage can be successfully treated with an intervention such as a stent which reintroduces vital blood back into the heart muscle. However, for a significant number of patients, interventions that restore blood to the heart muscle can paradoxically lead to irreparable and extensive muscle damage. This stops the heart muscle from pumping efficiently, putting the patient at increased risk of developing heart failure and can even cause death. Indeed, while more people than ever are surviving heart attacks, more people are living with heart failure. The poor functioning of the heart after an MI can also lead to other organs in the body getting damaged, which further increases the chances of high morbidity and mortality.

Increasing evidence, including our own, suggests the extensive damage to the heart muscle after an MI may be due to unresolved problems, not in the large blood vessels, but within the smallest blood vessels or 'microvessels' of the heart. The health of these microvessels is of utmost importance as they are ultimately responsible for delivering the oxygen and nutrients to the working cells of the heart.

However, microvessels are not visible to cardiologists even when using the most sophisticated clinical imaging tools (e.g. x-ray angiography can only visualise 10-15% of the blood vessels of the heart muscle). Problems that can take place in these microvessels include unwanted blood clotting and sticking of circulating blood cells to the vessel walls, narrowing of the blood vessels, leakage of blood fluid (plasma), damage to cells making up the blood vessels etc. Collectively, these various problems can impede the flow of blood and decrease the number of properly functioning microvessels, thus contributing to the overall poor health of the heart muscle after an MI. This can consequently lead to damage and the poor functioning of other organs in the body such as the lungs, gut, liver, kidneys etc.

There is a high prevalence of MI in individuals who are elderly or have type 2 diabetes mellitus (T2DM) or chronic kidney disease (CKD). Unfortunately, the outcomes after an MI for patients with these common co-morbidities is much worse.

For example, patients with T2DM and CKD are not only three times more likely to have an MI, but also subsequently suffer from greater damage to the heart muscle and higher rates of morbidity and mortality. It is possible that these co-morbidities or risk factors not only damage the microvessels of the heart, but also increase the susceptibility of these tiny blood vessels to greater damage after an MI. This poses a serious problem when considering the ageing population and the epidemic rise of T2DM and CKD worldwide.

Since it is not possible to see the microvessels of the heart in real-time in humans, the exact nature of the perturbations taking place within them after an MI, or in the presence of co-morbidities, is not known. Without this knowledge, we cannot develop strategies to prevent microvessel damage and consequently preserve the viability of the heart muscle in patients after an MI. Intravital microscopy is a sophisticated and powerful research tool that can be used for real-time imaging of even the tiniest blood vessels in terminally anaesthetised animals (i.e. animals under an anaesthetic from which they will not recover) and, therefore, under conditions closely approximating those of a natural environment. It will be applied to the mouse heart to look at the impact of MI on the microvessels of the heart in the presence of three common co-morbidities / risk factors, namely ageing, T2DM and CKD. Despite being recognised by the cardiovascular basic science and clinical community as a research priority, the processes that drive dysfunction within the microvessels of the heart are currently poorly understood.

Within the remit of our current licence, we have already discovered previously undescribed perturbations in the tiny blood vessels of the beating heart in experimental mouse models of MI. We have also identified some of the pathological mechanisms underlying these events. However, although valuable information has been obtained, there is still a lot to learn. Due to this knowledge gap, it is critical that we discover novel pathophysiological mechanisms specifically mediating problems in the microvessels of the heart that may translate into new clinical therapies. Testing for these in appropriate experimental models of MI, and in the presence of co-morbidities, is essential to develop therapies that, importantly, remain effective in patients with co-morbidities. Overall, this is important since MI in the elderly, diabetic and CKD population constitutes a major global health and economic burden on society.

Summary: Approximately 50% of patients suffering from an MI develop significant damage to the heart muscle which can progress to heart failure and even death.

This is despite successful opening of the large blocked artery(s) of the heart that caused the MI. These patients are predominantly those with co-morbidities.

Unresolved problems within the smallest blood vessels of the heart may contribute to this poor prognosis. However, these cannot be seen clinically by cardiologists.

Therefore, application of experimental techniques that allow the microvessels of the heart to be imaged is important as it will allow the development and testing of strategies that could potentially confer therapeutic benefit at the level of these tiny blood vessels and accelerate the much-needed clinical progress in this area.

What outputs do you think you will see at the end of this project?

- New data, knowledge and novel insights about the pathological role of the coronary microcirculation in myocardial infarction, particularly in the presence of common co-morbidities
- Open access publications in peer reviewed journals and published academic conference proceedings.
- Presentations at national and international basic science and clinical conferences and workshops.
- Presentations at academic institutions.
- Engagement with a wide variety of audiences including public engagement activities.

- Sharing of knowledge with patient groups and receiving their feedback on how we should further develop our research.
- Improved research methods for imaging blood vessels in vivo.
- Candidate compounds expected to be progressed to clinical trials.

Who or what will benefit from these outputs, and how?

In the short-term, researchers within and external to the Establishment will benefit from the new data, knowledge and novel insights about the pathological role of the coronary microcirculation in myocardial infarction and other ischaemic cardiovascular diseases. Researchers in other disciplines such as ageing, diabetes and renal diseases interested in how these co-morbidities can affect the functioning of blood vessels will also gain novel insights. In the medium-term, the wider research field will benefit from the published data containing this knowledge which will inform the direction of future research for treating cardiovascular diseases, highlighting specific and strategic areas to focus on. In the long term and beyond the scope of this licence, patients suffering from a heart attack will benefit for new therapies that can be developed. This is particularly true for the most vulnerable patients who are either aged, diabetic of suffering from an additional diseases such as CKD.

How will you look to maximise the outputs of this work?

- Open access publications and conference proceedings.
- Presentations at national and international basic science and clinical conferences and workshops.
- Presentations at academic institutions.
- Making resources (e.g. data, tissue) available to other researchers.
- Engagement with a wide variety of audiences including public engagement activities.

Species and numbers of animals expected to be used

- Mice: 7000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.
Explain why you are using these types of animals and your choice of life stages.

Mice will be the species of choice for all procedures. The age of mice will vary between approximately 8-weeks-old to 24-months-old. This is because it is increasingly recognised that the way blood vessels behave in both health and disease varies considerably between young and old mice, similarly to humans. This clearly has implications for the development of therapies that need to remain effective in elderly MI patients, the demographic most affected by MI, and where the susceptibility of the blood vessels to injury is greater.

To answer these important clinically relevant questions, we will use powerful *in vivo* imaging research microscopes to gain insights about a whole host of dynamic events taking place inside even the smallest blood vessel of the beating heart in terminally anaesthetised mice. These sophisticated imaging techniques permit direct and real-time

observations to be made in blood vessels in a truly physiological and pathological setting. Mice are the best vertebrate model for imaging these events as it would not be possible to conduct similar experiments on fish or worms for scientific or logistical reasons e.g. worms do not have a 'heart'. Mice are also the least sentient species in which the behaviour and responses of blood vessels to disease and injury is not only well characterised, but also similar to humans. For example, the mechanisms by which circulating blood cells of mice interact with the surrounding lining of the blood vessels during vascular processes such as inflammation or blood clotting are also shared by humans. Also, many responses we plan to study from a scientific viewpoint are best characterised in this species due to the widespread availability of defined transgenic mice and pharmacological tools that work in mice.

This is important as it avoids the need to use additional animals to re-establish baseline data in a different species. Importantly, the relevant scientific protocols are already well established and refined for mice.

Typically, what will be done to an animal used in your project?

- We will characterise disturbances taking place in the microvessels of the heart in **experimental mouse models of MI.** This will involve temporarily closing a large blood vessel (artery) in the terminally anaesthetised mouse heart to mimic the arterial blockage taking place during a heart attack in a human. The closure will then be released, allowing the heart muscle to be reperfused. This mimics the flow restoring procedures performed in the clinic (e.g. by the insertion of a stent). We will also look for disturbances in the blood vessels of other organs in the same terminally anaesthetised mouse to identify any possible adverse effects of an MI in remote organs. These experiments will be performed in either **adult or aged mice** which will allow us to test whether ageing increases the susceptibility of the microvessels of the heart to damage after an MI. All imaging experiments will be undertaken in the same mouse under the same non-recovery anaesthesia used to induce the MI.
- We will characterise disturbances taking place in the microvessels of the heart in **experimental models of MI in mice with type 2 diabetes mellitus (T2DM).** This will allow us to test whether existing chronic hyperglycaemia (high levels of blood glucose) damages these blood vessels and whether it increases their susceptibility to greater damage after an MI. Mice will typically receive diets for up to 16 weeks that will induce obesity-associated diabetes as this experimentally models the development of T2DM in humans. All subsequent imaging experiments will be undertaken under non-recovery anaesthesia.
- We will characterise disturbances taking place in the microvessels of the heart in **experimental models of MI in mice with chronic kidney disease (CKD).** This will allow us to test whether existing CKD damages these blood vessels and whether it increases their susceptibility to greater damage after an MI. Mice will typically receive a specialised diet for up to 20 weeks that progressively damages the kidney in a manner that resembles the CKD seen in humans. All subsequent imaging experiments will be undertaken under non-recovery anaesthesia.
- We will **investigate the pathways involved in mediating disturbances in the microvessels of the heart** in the presence of ageing, diabetes or CKD. For this, we may perform the above experiments in animals carrying genetic alterations in the pathways of interest. We may also administer pharmacological agents that directly impact these pathways. These will be delivered either via injection in conscious

animals, or during the final imaging step that takes place under non-recovery anaesthesia, depending on when the pathways need to be manipulated. All subsequent imaging experiments will be undertaken under non-recovery anaesthesia.

- We will **develop and test therapeutic treatments that can protect the microvessels of the heart** after an MI and assess whether they remain effective in the presence of an ageing, diabetic or CKD co-morbidity. We will administer therapeutic agents either via injection in conscious animals, or during the final imaging step that takes place under non-recovery anaesthesia, depending on how long these drugs take to be effective. All subsequent imaging experiments will be undertaken under non-recovery anaesthesia.
- • Some mice will undergo **routine blood sampling** and/or a large volume of blood will be taken under terminal anaesthesia. **Tissues may also be taken** post-mortem for further analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Imaging blood vessels: All imaging procedures in mice will be undertaken under non-recovery, terminal anaesthesia where the animals will only be aware of the anaesthetic being administered and hence no adverse effects are expected.

Aged mice: The use of aged mice may be associated with a potential for increased age-related aggression, anxiety and irritability, particularly in aged male mice. These typically appear by 18- months of age. Additional age-related effects that may be noted include weight loss, reduced activity, hunched posture, loss of muscle tone, coat thinning, skin lesions, palpable tumour formation and dental problems.

Diabetic and CKD mice: Some animals will undergo changes in their daily diet for 16 weeks to induce diabetes which are not expected to cause distress but will result in obesity and a greasy skin which can be accompanied by skin itchiness or irritation.
These animals will be closely monitored for emerging signs of this occurring. Some animals will undergo changes in their daily diet for 20 weeks to induce CKD which will result in weight loss due to unpalatability, increased urination, thirst and lethargy.

Pre-treating mice with pharmacological or therapeutic interventions: Some conscious animals may be pre-treated with pharmacological agents in order to understand the pathways mediating disturbances in the microvessels of the heart. Some animals may be pre-treated with therapeutic interventions in order to develop treatments that can protect the microvessels of the heart from injury. These may need to be delivered within 48 hours prior to the induction of non-recovery anaesthesia. This is needed to give these agents sufficient time to elicit their response. Typically, these animals will experience brief, slight discomfort and mild stress due to restraint and no lasting harm from administration of substances by injection using standard routes (e.g. intravenous, subcutaneous, intraperitoneal, oral). It is possible that with some of these agents, mice may exhibit signs of toxicity such as weight loss, subdued behaviour and piloerection but based on our extensive experience, the anticipated incidence of this is <5%. We will perform pilot studies in the event that novel agents are being used.

Blood sampling: Some animals will experience very brief discomfort from blood sampling associated with insertion of a small needle through the skin.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

5% - mice - sub-threshold 20% - mice - non-recovery 60% - mice - mild
15% - mice - moderate

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We aim to understand how disturbances taking place inside the smallest blood vessels of the heart contribute to the poor outcomes of patients after an MI, particularly high risk patients such as the elderly, diabetic and those with an existing chronic disease of a different organ such as CKD. Ultimately, our goal is to develop novel strategies to inhibit detrimental processes taking place within them and thereby protect these delicate blood vessels. Problematic events taking place in the blood vessels of the heart after an MI may include unwanted and deleterious interactions of circulating blood cells with the lining of blood vessels, unwanted blood clotting events, reductions in blood flow, narrowing of the diameter of blood vessels, leakage of vascular fluid (plasma) which causes tissue swelling etc. Our lab uses an array of non-animal based (*in vitro*) molecular and cell biology methods to understand the events underlying physiological, pathological and therapeutic processes taking place in blood vessels. However, these processes are so complex, with many occurring at the same time and mechanistically interconnected, that they cannot currently be replicated in any non-animal based system.

For example, the inappropriate recruitment, retention and activation of circulating blood cells within blood vessels can cause them to subsequently damage not only the blood vessels that captured them but also the heart itself. We hope to identify and then inhibit these processes in the aftermath of an MI. However, circulating blood cells rely upon their surrounding environment for cues or signals in order for blood vessels to capture and activate them. These signals can come from the blood vessels and circulating cells themselves, other non-vascular cells in the heart, the surrounding nerves, soluble circulating factors such as hormones etc. However, this entire living environment of the heart cannot all be incorporated in single or multiple cell co-culture assays. This makes it impossible to identify the exact deleterious pathways governing damage to blood vessels in the heart after an MI without the use of an animal model.

Furthermore, the degree of information about events taking place in blood vessels that can obtained from clinical tissue biopsy samples is limited. This is because these are one-time 'static' snapshots that cannot capture dynamic processes taking place in blood vessels.

For example, it is not possible to know whether circulating blood cells (e.g. a red blood cell or a white blood cell) observed in blood vessels in these histology samples were actually stuck to the lining of the blood vessels and potentially blocking the passage of blood, or whether these cells were circulating cells that were freely passing through the blood vessel at the time of tissue retrieval.

Circulating cells that are genuinely and inappropriately stuck are more likely to cause damage to the surrounding blood vessels and impede blood flow - hence, we need to be able to accurately assess their behaviour. Additionally, a heart attack may change dynamic processes such as the pattern of blood flow, the velocity of blood flow and the diameters of blood vessels, or damage the blood vessels and make them more leaky.

However, information about these dynamic vascular processes can only be obtained from living tissues and organs. These cannot be imaged in the standard ‘static’ vascular research assays. Moreover, how diseases and injuries modify dynamic processes taking place in blood vessels varies depending on the tissues or organs being investigated as well as the type of disease itself. For example, a heart attack may modify blood vessels in a different way to how liver cancer may modify tumour blood vessels. This makes accurate modelling of these site-specific vascular events impossible *in vitro,* necessitating the use of appropriate experimental models of disease in living animals.

It is also difficult to accurately reproduce *in vitro* the exact and complex nature of the environment that surrounds blood vessels in patients with co-morbidities such as age, diabetes or CKD. For this, we would need to look at events taking place in aged mice or utilise experimental models of mice with diabetes or CKD. How the presence of an existing chronic condition such as CKD in a completely different organ (i.e. the kidneys) subsequently leads to damage to remote microvessels in the heart is also difficult to reproduce *in vitro* and requires the whole body to establish these multi-organ pathologies.

Therefore, we need animals to achieve the aims of our project.

The heart blood vessels will be imaged using our sophisticated and powerful imaging microscopes which offers the best method for evaluating dynamic vascular events as they permit direct and real-time microcirculatory dynamic observations to be made in a truly physiological and pathophysiological setting.

Which non-animal alternatives did you consider for use in this project?

We have considered, and do use where appropriate, a multidisciplinary array of *in vitro* molecular and cell biology methods to understand events underlying physiological, pathological and therapeutic processes in the vasculature. These include single and multiple cell co-culture assays, static and flow based adhesion assays, histology and flow cytometry.

Why were they not suitable?

We can use non-animal *in vitro* assays (e.g. cell culture and cell adhesion assays) to grow the lining of blood vessels and then assess the interactions of isolated circulating blood cells. For example, we can use these assays to understand how circulating blood cells (e.g. white blood cells) inappropriately stick to blood vessels and try to prevent this from

happening. This is important to understand because if this happens in the tiny blood vessels of the heart, they may hinder or block the flow of blood within them depriving the heart of essential oxygen and nutrients. However, it is not possible to replicate in these assays all of the surrounding environment that these cells are exposed to in their natural setting. This is critical as circulating blood cells and the cells that make up the lining of blood vessels receive cues or signals from their environment to guide their behaviour in animals (*in vivo*). These important cues can also come from the flow of blood itself as well as from neuronal and hormonal influences, all of which cannot currently be incorporated into our *in vitro* assays. Moreover, we cannot fully replicate the complex spatial and temporal interactions that take place within blood vessels during various diseases and injuries, all of which can influence vascular events. Nor is it possible to entirely replicate the many additional complications that are associated with co-morbidities such as ageing, diabetes and CKD. Whilst imaging clinical tissue biopsy samples histologically is useful, the degree of information about processes taking place in blood vessels that can obtained from these one-time static snapshots is limited, especially for dynamic events. Moreover, important information about how blood vessels are working or functioning can only be obtained from living tissues and organs e.g. has the disease or injury changed the pattern of blood flow or the diameters of blood vessels or made them more leaky.

Nevertheless, we do use these various non-animal alternatives to guide and inform our animal work and therefore, importantly, these *in vitro* techniques continue to have an important role to play in our project.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Experimental design methods have been employed to ensure that the right type of data, and enough of it, is used to answer the questions of interest as clearly and efficiently as possible. We have been performing similar experiments for over 30 years and the data collected has been subject to statistical analysis using appropriate software and with the help of a statistician. This has allowed us to calculate the minimum numbers of animals that need to be used whilst ensuring that the results are statistically significant. For new experiments, we perform pilot studies to estimate standard deviations of groups and this data is used to calculate optimal animal numbers for definitive experiments. Our previous experience, and that of other researchers conducting similar experiments, typically show that we need groups sizes of 6-8 mice to achieve the quality of results that we need with sufficient power for appropriate interpretation.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have used the NC3Rs' experimental design guidance and experimental design assistant to plan our experimental design and statistical analysis and will continue to do so

for new planned experiments. We have also sought expert statistical advice and referred to our previous experience.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Image multiple vascular events in the same animal: We use sophisticated imaging microscopes to visualise a variety of events taking place inside the blood vessels of mice.

Our set-up is state-of-the- art and allows more than one event to be captured at the same time in the same mouse. Over the years, advancements in imaging technology have allowed us to dramatically reduce the numbers of animals needed to get the same data.

For example, we can image a circulating white blood cell at the same time as imaging the leakage of blood fluid (plasma) from damaged blood vessels. Therefore, fewer animals need to be used to generate more valuable data. Indeed, up to three different events and/or anatomical components of blood vessels can be imaged at the same time in the same animal. Importantly, this allows more meaningful spatial and temporal correlations between different events and components of the blood vessels to be made.

Maximise the information gained from a single experiment: To ensure as much data and information can be obtained from the same mouse, where possible, we take samples and measurements from multiple body sites. For example, in addition to imaging the blood vessels of interest *in vivo*, we may take blood pressure / heart rate measurements, blood samples and tissue samples from the same mouse. To look at the adverse effects of an MI on the blood vessels of remote organs such as the lungs, gut, liver, kidneys etc. we can image these organs in the same terminally anaesthetised animal, which will again maximise information that can be obtained from the same mouse. At the end of the imaging experiments, we will harvest as many tissues as possible at post- mortem. If we don't need to analyse the tissues immediately, we will freeze them and make them available to other researchers working on similar questions or disease models.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The majority of techniques conducted under the remit of this licence will be performed under terminal anaesthesia and so pose no pain, distress and suffering to the non-recovering animal beyond the event to induce anaesthesia. This includes experimentally modelling an MI in the mouse. During an MI, a blockage occurs within a large blood vessel (artery) in the heart. This can subsequently be opened using clinical interventions such as a stent. We can model this in the anaesthetised mouse heart by temporarily tying a suture

around a large artery of the heart to mimic the period of time when the heart muscle was deprived of blood during the heart attack. We can then un-tie this suture to mimic the clinical restoration of blood flow.

This experimental model of an MI is well characterised, highly reproducible and can be easily induced in terminally anaesthetised mice. The damaging events taking place in the microvessels of the beating heart in the aftermath of an MI will then be microscopically imaged over a period of up to 6 hours and therapies will be designed and tested to prevent them. This procedure is performed under terminal anaesthesia and so there is no pain, suffering and distress to the non-recovering animal.

Some animals will need to undergo procedures prior to the induction of anaesthesia to achieve our scientific needs. For example, if we believe a certain compound (e.g. a specific type of inflammatory protein called a cytokine) may be mechanistically mediating the observed perturbations in the blood vessels of the heart, we will administer this individual compound to see if it elicits responses in these blood vessels that are similar to an MI injury. However, certain pharmacological agents may need to be administered through an appropriate route of delivery up to 48 hours prior to terminal anaesthesia to give them sufficient time to elicit their acute response in the microvessels of the heart. Similarly, any therapeutic agents we are interested in may also need to be administered through an appropriate route of delivery up to 48 hours prior to terminal anaesthesia to give them sufficient time to elicit a beneficial effect. The specific agents to be used are well-characterised by us and others for their mild effects in mice. By using well established protocols to elicit responses in the blood vessels of the heart, we can minimise any unknown effects on the mice and subsequently minimise or prevent pain, distress and suffering.

To test the impact of common co-morbidities on the microvessels of the heart and also subsequent to an MI, some mice may either be aged, made diabetic or have CKD. In humans, T2DM is brought about primarily through obesity and poor diet.
Feeding mice well established and characterised commercial diets that are high in fats and sugars is a model that has been refined over the years to closely mimic the progression of T2DM in humans. Indeed, this experimental approach leads to the development of obesity and also demonstrates clinical features seen in patients with diabetes (e.g. impaired glucose tolerance, increased insulin secretion, increased insulin resistance). We will feed these diets to mice for up to 16 weeks. This will cause obesity and a greasy coat which can be accompanied by skin irritation.

However, this model is more refined that other experimental models of T2DM, such as combining a high fat and high sugar diet with chemicals such as streptozotocin or alloxan, because these chemicals have off-target side effects and interfere with other biological functions in mice complicating the interpretation of results. Some mice will have this diet reversed back to a normal chow diet which is not known to cause any pain, suffering or distress to the animal, and in fact reflects a return towards a healthy body condition.

To induce CKD, mice will receive dietary supplementation with a molecule called adenine to induce a gradual and persistent reduction in kidney function. These changes imitate the pathological process of CKD in humans. The effects of adenine on the kidney are controlled by dose and duration of adenine exposure. This allows for the fine-tuning of the level of kidney impairment required for the planned final imaging experiments that will performed under terminal anaesthesia. Furthermore, it means that changes for the animal

are very mild early on, building only gradually over time. Hence, the animal experiences more significant changes only for a limited duration. Effects of adenine are very specific for the kidney as it becomes concentrated there. Therefore, direct harm to other organs by adenine is avoided.

This diet-based model of CKD is non-invasive and therefore more refined than the alternative surgical-based models e.g. removing one whole kidney and more than half of the other followed by recovery. Also, it has a high success rate and animal losses that would occur because of procedural / surgical complications or variation in the surgical skill level of the researcher are avoided. Importantly, it spares the animal from the distress and traumatic recovery period related to surgeries.

Why can't you use animals that are less sentient?

Non-mammalian animals are limited in their use when studying the response of blood vessels and the actions of the circulating blood cells within them (both inflammatory and immune circulating blood cells). Indeed, non-mammalian animals do not have the right type of circulating immune cells and their immune system is too different from the human immune system to provide relevant results.

Furthermore, it would be challenging, if not impossible, to conduct similar experiments on fish or worms as they do not have a cardiovascular system similar to mammals e.g. worms do not have a 'heart' as such. Lesser developed species (invertebrates) or aquatic life forms either do not possess the organs we wish to study or they do not resemble human organs and are therefore not suitable.

Due to the models required and the time involved, we need to use adults, but where possible we will perform techniques under terminal anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Refinements when using aged mice: Aged mice will be flagged for increased monitoring as they turn 18-months-old to check for clinical signs of ageing which give rise to adverse events if cumulative or if left unchecked. An ageing score sheet will be kept by the local animal facility staff and/or researchers and regular weighing will be instigated where appropriate. The score sheets will be established in conjunction with advice from the NVS and NACWO to determine humane end points for clinical signs due to ageing in addition to the humane endpoints already outlined for the procedures to be undertaken in the protocol. If ill health is detected a number of routes may be taken. If animals develop age-related skin sores, then barrier creams may be used to achieve resolution. If there is increased prevalence of barbering or repetitive behaviours then additional enrichment activities or altered housing conditions will be used to improve psychological welfare. To mitigate the potential for aggressive male behaviour during ageing, which may lead to the need for single housing, stable social groups will be established from an early age, and additional/varied enrichment will be provided. For males unable to be housed with other male mice, suitable companions may be sought that do not induce male behavioural changes and mitigate isolation stress (e.g. sterile or menopausal females).

Refinements when using mice with diabetes or CKD: When using the experimental diabetes and CKD mouse models, we will draw on our personal experience (e.g. for the

diabetic mouse model), work closely with both local and national researchers who are experts in these models, as well as refer to published work for up-dated guidance on improving animal welfare and refinements in protocols.

For example, the coats of mice being fed the high fat diets to induce diabetes will be carefully monitored. Furthermore, their diet will be provided on the floor to avoid it dropping on to their fur and exacerbating the issue with greasy coats. Soft bedding will be used to avoid skin irritation. If skin irritation occurs it will be treated using an anti-inflammatory cream. Mice on this diet, who are returned back to a normal chow diet, will be weighed first and their body condition, behaviour and activity monitored.

The CKD mouse model involves development of renal impairment which is expected to increase urine production and thirst. Mice who are urinating excessively will be provided an alternative bedding that is more absorbant and only up to 4 animals will be housed per cage. Animals will be monitored using bespoke clinical score sheets for developing adverse effects that includes the recording of both body weight and body condition. In addition to the adenine diet in the cage hopper, diet will be placed in a readily accessible location on the cage floor to encourage feeding.

Refinements when delivering pharmacological and therapeutic interventions prior to terminal anaesthesia: Some procedures to deliver pharmacological or therapeutic agents will be performed prior to the induction of terminal anaesthesia. Trained personnel will perform such procedures using refined handling techniques to minimise any distress to the animal (e.g. when performing oral gavage). If pilot studies are required, animals will be closely monitored for the first 30 minutes after administration, then at least twice a day for signs of developing adverse effects.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

There are no specific best practice guidelines for the procedures to be used on on this project, many of which have been developed and optimised for bespoke use in this laboratory and those of local researchers. However, monitoring of the ageing animals will be based upon best practice guidelines for ageing which are available at: **Wilkinson et al., Progressing the care, husbandry and management of ageing mice used in scientific studies. Lab Anim. 2020; 54(3):225-238.** More general best practice guidelines (e.g. for husbandry or blood sampling) provided by the NC3Rs will be followed, as will the recommendations of the various local animal facility staff and national committees on the refinement of procedures for administration of substances. We will also follow guidelines provided by ARRIVE 2 (**https://arriveguidelines.org/arrive-guidelines**) and LASA (**https://www.lasa.co.uk**/).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will remain in close dialogue with local animal facility staff, the Named Veterinary Service (NVS) and the Named Animal Care and Welfare Officer (NACWO) to extend our knowledge about general and specific welfare issues relating to the experimental animals. We regularly receive the NC3Rs e- newsletter and check the NC3Rs website for updated guidance on common procedures. Furthermore, we work closely with other researchers using similar models to advance our knowledge of animal welfare and 3Rs efficiently.

Finally, we regularly review the relevant literature, attend professional research conferences and participate in animal research training opportunities to achieve continuous professional development on any relevant advances and animal husbandry in general. Where necessary, we will perform pilot studies to gather information and improve the quality and efficiency of subsequent animal experiments. We will publish our *in vivo* data in open access journals and in accordance with the 'Animals Research Reporting of *in vivo* Experiments (ARRIVE) guidelines for reporting. Collectively, these efforts will ensure that we remain continuously up-to-date on the highest welfare standards specifically relating to our specific animal models and promptly refine procedures wherever possible.

48. Characterising the role of post-translational modifications and specific gene modifications on innate immunity in mouse models

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Immunology, Phagosome, Signalling, Infection

Animal types	Life stages
Mice	juvenile, adult, pregnant, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project will study how specific gene and protein modifications (changes occurring to proteins following protein production) can affect the immune system using mouse models deficient in certain genes or proteins (proteins being one of the major building blocks of the human body, and keeps the immune system functioning).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Infectious diseases, cardiovascular (heart and vessel related) disease, diabetes, autoimmunity (overactive defence system causing harm to the body) and cancer affect a high percentage of the UK population. In particular, immunity has been shown to contribute to many of these diseases, for example autoimmune diseases, affect more than 6 % of the

UK population.

This animal research project will contribute to the expansion of knowledge and understanding of possible immune related problems which may aid in future translational and therapeutical (producing treatments such as new medicines) efforts globally.

What outputs do you think you will see at the end of this project?

Our animal work is basic research, which aims to further our understanding and knowledge of the mechanisms behind some immune related diseases. Our ongoing animal work has contributed to the identification and publication of the roles and mechanisms of multiple proteins linked to inflammatory diseases.

Our main focus will be on the biology of a type of white cells that kill micro-organisms (macrophages) and how cell membrane forms and envelopes these micro-organisms (phagosome). Macrophages are important innate immune (non-specific defence) cells that play major roles in a large number of processes, including metabolism (reactions that provide energy and produce new essential materials in the living cells) and chronic inflammation (response by defence system that lasts after resolving the main cause of injury) in obesity (abnormal overweight), fatty liver disease, allergy (a condition that makes a person become sick or develop skin or breathing problems because they have eaten certain foods or been near certain substances) as well as bacterial and fungal infection (pathogens).

Macrophages are very effective at surrounding and killing micro-organisms, ingesting foreign material and removing dead cells during development of normal tissue functioning and infection by pathological micro-organisms (microbes) during infection. Once a micro-organism is internalised into the macrophage, newly formed phagosomes further develop with other cell contents (lysosomes) to breakdown the foreign material. Microbe (degradation) breakdown in the phagosome produces
antigens (substances that active the production of antibodies) on the cell surface to activate immune responses, linking general immunity (innate) to specialised immunity (adaptive).

The research will be carried out to study the role of specific gene/proteins: MSR1, RNF115, RNF213, DTX3L and ISG15, in innate immunity and lysosome function. We will characterise the role of these protein and possible downstream modifications on innate immune functions in macrophages and other immune cells. This will provide us with a fundamental understanding of the role that these genes/proteins play in the immune system.

These proteins play an important role in the defence against infections by altering the activity of macrophages. There is preliminary data about their function but little is known about the regulation of their function in the context of these cells and on systemic immune response (defence response which may affect the whole body).

Here, we will explore whether specific proteins affect the ability of the body to defend itself against dangers such as bacteria, using different parameters such as bacterial survival, changes to white blood cells, etc.

We will continue our contribution to better scientific knowledge via published articles

including positive and negative effects of our new targets in the immune system in high impact papers, posters and presentations at national and international conferences. These findings may contribute to the development of detection tests and treatments for some diseases.

Who or what will benefit from these outputs, and how?

Our animal work will mainly work towards scientific advancement in the short-term; in understanding the essential role of post-translational modifications (changes occurring to proteins following protein synthesis, their production) that could have a huge effect on protein function and activity) and specific gene modifications in innate immunity (non-specific defence). Thus in the long-term, this will support the global research community in better understanding the role of innate immunity in some diseases which may lead to the future development of better potential targeted therapy or precision medicines.

How will you look to maximise the outputs of this work?

The knowledge achieved through our results will be disseminated via presentations at conferences, via research publications in open-access high impact journals (reporting relevant experimental details effectively using both positive and negative data). We have an extensive collaborative network for which our data, stemming from this work, can be shared, and built upon by the wider research community.

Additionally, through publication we upload our datasets to online repositories so that anyone can access and view this data.

Species and numbers of animals expected to be used

- Mice: Protocol 1 will generate 11500 animals and 3500 from other projects

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The animal work to be carried out in mice aged between seven weeks and five months (juvenile to adult), will be divided into two components.

- First, mice will be used as a source of material for primary cell or tissue culture *in vitro* , mostly for the generation of bone marrow-derived macrophages (white blood cells that help eliminate foreign substances). These will be invaluable to allow the study of what happens on the cellular level as a results of specific treatments to test the defence system. It is expected that the majority of the work to be carried out (85-95%), will fall into this first category, and this approach will be used whenever possible in order to minimise the need for experimental interventions in live animals.

- Cells in culture do not communicate in the same way as they do in live animals . In order to overcome this limitation. it will thus be necessary to perform experiments using

live animals, *in vivo*, and test the effects of a knock-out (remove) of specific proteins involved in the immune system. This will form a smaller (approximately 5-15% of animals will be required to achieve this) component of the project and will consist of directed experiments to test specific hypotheses, using the infection or injection of inflammatory agents or the use of inhibitors/activators of specific molecular pathways (chemicals used to reduce or increase specific activities on cell level). This component is absolutely necessary if we want to expand and apply our research beyond in vitro experiments which are not typically representative of true physiological conditions (I.e., the immune response).

Typically, what will be done to an animal used in your project?

More than 80% of genetically modified animals (GMA) will experience mild severity as they will only be bred using standard breeding practices (natural mating) and be genotyped using the least invasive methods available, such as ear biopsy. Tissue collection for *in vitro* experiments will require some GMA animals to be humanely euthanized.

However, in up to 20% of the experimental studies (including GMA and wild-type animal as control for the experiments), animals may experience mild to moderate severity as they will undergo experimental procedures including either:
Bacterial or viral infection via an appropriate route as up to two administrations, with blood sampling occurring up to four times during the study, followed by humane euthanasia of the mice which will be used to collect tissues later up to seven days later.
or
An injection using an inflammatory compound/drug into the intraperitoneal (abdominal) cavity as up to two administrations. This procedure will help to recruit circulating monocytes (a type of white blood cell) that differentiate (the process by which certain cells transform into another, more specific,cell type i.e., monocytes can become macrophages or dendritic cells) into peritoneal macrophages, then humanely euthanised mice will be used to collect tissues up to seven days later.

What are the expected impacts and/or adverse effects for the animals during your project?

Breeding GMA animal should experience normal mouse behaviour and should show no harmful defects.

Bacterial/ viral infected animals may develop clinical signs depending on the pathogen after 7-20 days post-infection such as; transient (temporary) weight loss, subdued behaviour e.g pilo erect fur and hunched posture, splenomegaly (a condition where the size of the spleen is increased) and enlarged liver - this is usually observed post-mortem, symptoms may last from 3 days post infection to humane end point of the experiment (up to 7 days post infection). Daily observation of the infected animals will be done by PIL holders and the institute technicians.

To achieve the scientific output regarding the effects of missing a specific protein within the immune response to infections. Tissue will be collected from infected animals on the fifth to seventh day post infection (the earliest point to avoid any extra suffering without losing the scientific aim). If any unexpected adverse effects are noted post infection, PPLH will determine support for the concerned animal(s) as well as the decision of euthanasia. As and when the concerned animal do not improve after support/ interventions, the mouse will

be humanely culled.

We do not expect any adverse effects after injection of thioglycollate. Specifically, since the injection will be administered into a body cavity that does not contain any organs, no adverse effects are expected. Despite this, mice will be monitored twice daily, morning and afternoon, for any signs of adverse effects. Specifically, we will observe for any signs of swelling/inflammation, radiating heat, or redness at the injection site.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The 90% of GMA, and 90% of wild type, animals will be in maintenance and breeding will be subthreshold to mild severity.

5% of GMA and 5% of wild type animal could experience mild severity following inflammatory injection 5% of GMA and 5% of wild type could experience moderate severity following bacterial/viral infections.

What will happen to animals used in this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The use of animals in this project is necessary for several reasons as following:
We use cell culture systems to study how changes in genes will affect how cells function. But the macrophages (one of the main white blood cells used in our study), can only survive for a short time in culture. So, we often use immortalized cells (cells have been altered therefore these cells can grow and live longer in culture) such as RAW264.7, J774A.1 or BMA3.1A7 macrophages.

Due to the nature in which immortalised immune cell lines are created (creation of mutations that allow the cell to proliferate, increase in number through division, indefinitely); they oftentimes do not behave similarly to their non-immortalised counterparts; especially so when dealing with immune cells.

Moreover, we characterised immortalised macrophages, showing that many important pathways in phagosome (a cellular organelle formed around a particle eaten by macrophages) biology are altered between bone marrow-derived, primary macrophages and cell lines.

Therefore, primary cells from humanely euthanised animals will be essential, and thus repeated isolations are required due to the short life span of primary macrophages in culture.

Primary immune cells are difficult to transfect (to make a change on the genetic level by introducing foreign nucleic acids, the naturally occurring chemical compounds that serve as the primary information-carrying molecules in cells),_ making it challenging to specifically disrupt the functions of the signalling systems being studied. The limited life span of primary cells makes it more challenging to target specific genes in culture experiments. These problems can be overcome by using gene targeting to generate knock-out mice, which can then be used to isolate immune cells with the necessary mutations.

The second stage of this work will require the testing of hypotheses made from the initial cell culture models in an *in vivo* situation. This work will be necessary in order to extend the finding into the much more complex situation which occurs *in vivo*, due to the requirement of multiple cell types to co-operate in order to control immune cell function.

At present it is not possible to recreate these complex systems in cell or tissue culture experiments. This work will be hypothesis driven, rather than screening of multiple mouse lines through an *in vivo* model to reduce the animal use to the absolute minimum.

Which non-animal alternatives did you consider for use in this project?

There are different cellular models to study the immune and inflammatory responses such as RAW264.7, J774A.1, BMA3.1A7 macrophages, and to reduce the number of animals used in this project, we considered the immortalisation of primary BMDM (bone marrow derived macrophages). The immortalisation procedure has shown to have an impact on cell response when compared with primary cells, to overcome this possibility, primary cells will be required for validation of the scientific outcome.

There are other non-animal alternatives that we considered, such as human macrophages derived from monocyte cell lines such as THP-1 (leukaemia monocytic cell line) and U937 (a cell line obtained from a patient with histiocytic lymphoma); However, previous publications have shown significant differences between these cell lines and primary human monocyte derived macrophages indicating these cell lines may not behave like primary cells in specific scientific challenges. The previously established literature, as well as our own findings, have shown that these cells, most likely due to being derived from cancer cells, do not behave like primary macrophages based on proteomic and flow cytometry data.

Human macrophages derived from primary monocytes, induced from stem cells, or obtained after human hip replacements and biopsies procedure, can be a non-animal alternative. In our lab, we aim to use human stem cells and to increase efficiency via finding alternative methods to produce the required growth factors for this process.

However, usually not enough cells can be harvested to conduct a scientific experiment (It takes up to six months to have enough cells for small experiments. In addition to that, the resulting primary cells are delegate and fragile and they will not survive any transfection.

All of these factors limit the use of these primary cells to understand the role of specific

proteins in immunity; therefore GA mice for primary cells is still unavoidable.

In addition to that, these cellular models have limitations since they are not able to contribute knowledge regarding the response resulting from communication between different immune cells and the effect on organs and other complex systems, therefore, limited in vivo work will be still vital.

Why were they not suitable?

Immortalised immune cell lines do not provide a good model as they do not respond in the same way as primary immune cells to specific stimuli (e.g. pathogen) and contain multiple mutations due to the immortalisation process. We have recently characterised these cell models and identified a lack of comparability to primary immune cells.

Primary immune cells isolated from humanely euthanised genetically modified animals are essential to study signalling on a cellular level.

The recreation of the complex environment within living organisms, where there are multiple cells and other cell types interacting together, such as cell-cell communication and the secretion of signalling molecules is extremely difficult to achieve. The absence of these signals in cell culture models could have an important impact on scientific results.

Mouse models will allow us to study the innate immune response to inflammatory disease incited by viruses or bacteria.

These points highlight the need for the use of experimental animals to validate these scientific results.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Approximately 3000 animals per year; maximum 15000 per 5 years
The majority of the work to be carried out will require the maintenance of colonies of genetically modified mouse lines in order to produce the number of animals required to set up primary cell culture models. Inbred background strain approximately every five generations will be required to maintain the lines on the correct genetic background. This will help keep the colony as genetically similar to the control as possible and ensure that results are reproducible.

Breeding programs will be reviewed in combination with the individual scientists responsible for the work program in order to ensure animals are produced in the correct numbers and at the right times. For the generation of experimental animals, we will use the most efficient breeding strategies to produce the required number of genetic modified and

control animals.

As the majority of work can be done using tissues harvested from genetically modified lines bred on this licence, relatively low numbers of animals are required for *in vivo* models, thus minimising the need to put animals through potentially painful procedures.

Previous work will dictate these breeding strategies, in addition to the discussion with the colony manager to have the least number of animals to have valid scientific output.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

One of the approaches to obtain primary mouse macrophages is from bone marrow progenitor cells and through factors that differentiate them into macrophages.

Although the protocol was standardized by many laboratories, ours being one that has contributed to improving this protocol, there are still limitations regarding the number, and biological significance, of macrophages obtained per mouse.

Peritoneal macrophages, which can be isolated from the peritoneum following inflammatory challenge, are much more biologically relevant as they are important for wound healing and dampening the immune response following inflammation.

Specifically, they are important for a process called efferocytosis: the removal of dead cells from specific tissue. Unfortunately, under normal conditions these peritoneal macrophages are almost non-existent. and so we would need approximately 10 times the number of animals to isolate the same number of peritoneal macrophages. This would not be ethical, and so in order to effectively study efferocytosis ex-vivo, we will use a common method to isolate these cells.

Animals will be injected intraperitoneally with thioglycollate solution, to facilitate the recruitment of immune cells to the peritoneal cavity, which can then be collected after culling mice no later than one week after thioglycollate injection. Ultimately, thioglycollate injection will enable us to use much fewer GMA and WT mice to achieve the same scientific result (previous publication have shown at least twenty time more cells could be collected after single injection with thioglycollate and this injection does not cause any side effects). Therefore, the harm could cause by the injection of thioglycollate is outweighed by the benefit of minimising the number of animals which required to have the same scientific output. We have optimised the protocol for isolating maximum number of cells from each mouse.

For *in vivo* models, group sizes will be sufficiently calculated to generate scientifically and statistically significant results. Where appropriate, group sizes will be based on previous experience with the model to be run, and this information will be used for power calculations to determine the minimum group size required. In order to achieve a statistical power of 80-90%, n=6-10 animals will be required. Otherwise, preliminary experiments using 4 to 8 animals per group will be carried out, and the results of these used to establish the required numbers via power calculations.

Where appropriate, the final data set will be achieved by combining the results obtained from a series of smaller blocks, rather than running a large number of animals through the

model simultaneously.

When this approach is used, appropriate controls will be included in each separate block, for example, to guard against potential confounding factors such as genetic drift in either the genetically altered or the “normal” mouse lines. Also, backcrossing can reduce the number of spontaneous mutations that may have accumulated.

The NC3R's Experimental Design Assistant will be used to model experimental group sizes, or to support with study planning.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Working with the colony management team, refinements in breeding and maintenance of GMA animals will be made by:
Cryopreserving animals where appropriate e.g. earliest opportunity after import of new strains or after backcrossing to ensure strains are preserved with a minimal amount of genetic drift and therefore preserving their integrity.

Backcrossing of mouse strains at appropriate points such as every 5-10 generations, dependent on colony size and backcrossing of both sexes to ensure good refresh of both sex chromosomes. This will preserve genetic health and the increased reproducibility by limiting genetic drift that occurs naturally across generations.

Utilising Single Nucleotide Polymorphism (SNP) analysis to ensure correct back crossing to preserve genetic health. Analysis will occur either when animals are frozen and/or before backcrossing occurs to establish the correct choice for background. In exceptional circumstance backcrossing will not be appropriate and working with the colony management team we will find alternative approaches to support preservation of genetic integrity.
Utilising the breeding system that is most suited to the efficient production of that strain of mouse, this will be based on data or information available in mouse passports e.g. pairs/trios.

Active monitoring of defects in breeding colonies will occur with support from the colony management team and technicians. This will identify early reoccurring health defects that may indicate genetic drift. Likewise, we will remove non-standard animals from breeding programs.

We will adhere to the local published standards set out by AWERB. These include: Rodent 12 Month Breeding Age Limit and Rodent Breeding Defect Management.

The local published standards are based on the standards set out in the ASPA and its associated codes of practice.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice will be handled with Hurst's non-aversive tube handling technique to reduce stress in animals.

For any of the protocols within this licence, animals (or just their tails) may be warmed prior to blood sampling using a method that has been agreed with the NVS and is not expected to cause any pain, suffering, distress or lasting harm. Warming will be used where this is considered to be a refinement in terms of reducing the overall stress for the animal in order to obtain a sample of the size required by the science (within LASA guidelines) and/ or where it allows fewer animals to be used.

For animal, microbial challenge (to administrate the bacteria to the animal and check their defence system after), the most appropriate route will be used, e.g. by oral, intra-nasal, oropharyngeal, intra- rectal, intradermal, intravenous or intraperitoneal routes using a single administration, and where appropriate, we will use anaesthesia for restraint, e.g. when microbes will be administered by intradermal skin prick of the ear. In addition, daily minimally invasive clinical monitoring and a scoresheet will be used to manage adverse effects and end points during bacterial/viral challenge experiments with the lowest severity possible to answer the scientific questions.

Most of the mice used in this project (at least 85% of all the GMA) will be used for breeding or the isolation of tissue, and therefore the numbers used in the interventional protocols will be relatively small.

In all protocols, animals will be killed via schedule 1 methods unless the quality or quantity of tissue required is such that it cannot be obtained using schedule 1 methods. In these case appropriate terminal procedures will be used, e.g. perfusion fixation to allow cells to be rapidly fixed and preserve quality, or exsanguination (death caused by loss of blood) to collect a large and non-haemolysed (red blood cells are not broken) blood sample.

Why can't you use animals that are less sentient?

Fish and insects (e.g. flies) lack the same broad range of immune cells found in humans, which means that there are differences between fish and mammals that could affect the disease biology.

Some genes are not conserved between these species and mammals, therefore some of the disease mechanisms may not be the same.

Therefore, drugs that target those cells or disease pathways may not work in these systems due to fundamental differences in the biology of the species compared to humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will use low stress handling for all animals, and all of the team will complete appropriate training and competency assessment for procedures.

In collaboration with the Named people, we have introduced increased monitoring for animals showing clinical signs (using a local score sheet)

When designing in vivo challenge experiments: administration route of microbes will be performed under the most appropriate route, using anaesthesia when required, and monitoring their clinical state will be achieved using the most minimally-invasive methods (eg. weighing, seeing their social behaviour, cleanliness, posture, skin hydration, etc.). Animals will be killed via schedule 1 methods.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Alongside the guidelines listed below, I will also adhere to local AWERB standards for research animals, and where appropriate, support the development of new local standards for refinements discovered during the project licence.

Code of Practice for Housing and Care of Animals Bred, Supplied or used for scientific purposes
NC3R`s Guidelines for administration and withdrawal of substances
RSPCA Animals in Science guidelines
UFAW Guidelines and Publications
NC3R's and Procedures with Care
Plus any additional

I will consult with the Colony Manager to review genetic health, breeding practices and overall colony health and management at regular intervals.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The local AWERB, NIO, NACWO, NTCO and Veterinary team regularly inform, and disseminate improvements and recent studies involving reduction, replacement and refinement.

Alongside external resources including (but not limited to); collaborators, peers, conferences and lab animal and animal welfare bodies.

During the 1-, 3- and 5- year review of the project licence, I will update on implementation or consideration of the 3Rs that has occurred during the previous period, alongside a review of the linked training plan, score sheets etc. in collaboration with the NACWO, NTCO, NIO and Veterinary team with a particular focus on refinements.

49. Creation and validation of a new model of Alzheimer's Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Brain, Neurodegeneration, Alzheimer's, Mice, Cognition

Animal types	Life stages
Mice	adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To generate and validate a new animal model of Alzheimer’s Disease, and to assess potential new therapeutics.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Effective treatments for Alzheimer’s disease, which target the disease process rather than the symptoms, are lacking. Animal models are vital tools in understanding disease pathology as well as in the development of treatments. Currently, however, the best animal models for Alzheimer's Disease fail to display the full range of symptoms and neuropathological hallmarks that are expressed in patients.
This may go some way to explain why current treatments fail to stop progression of the

disease. With a better animal model, we will perform proof-of-concept studies to investigate innovative and more effective therapeutic agents to treat Alzheimer’s Disease.

What outputs do you think you will see at the end of this project?

A new animal model of Alzheimer's Disease

The primary output will be a new validated animal model of Alzheimer's Disease, which will include assessment of brain biochemistry and histopathology. It will also validate the model using an appropriate range of behavioural tests; importantly, this will not only include tests of learning and memory, the most visible symptoms of Alzheimer's Disease, but also more subtle 'pre-cognitive' symptoms that occur before deficits in learning and memory appear. These symptoms include anxiety, sleep disturbances, depression, and reduced motivation to engage in rewarding behaviours. This stage at which pre-cognitive symptoms arise is an important point in disease development because if such symptoms can be detected in our mouse model, and treatment delivered at this early stage, it offers the opportunity of halting disease progression before the worst symptoms arise. Thus, a better animal model will allow development of drugs that can be used earlier in the disease progression; and the earlier the diagnosis in human populations, the earlier potential treatments can be tested and started.

The work will also lead to a better mechanistic understanding of the causes of Alzheimer's Disease, as well as the learning processes that are dysfunctional. With methodology shared with other groups, this understanding could also be informative for other neurodegenerative disorders and therapeutic areas.

It is anticipated that several publications (both peer-reviewed primary research and review articles) will be produced by the end of this project. Data may also be presented at international conferences.

Therapeutics and biomarkers

The project will allow further validation of an Alzheimer's Disease therapeutic, which would be further developed for future clinical trials. We will also generate a significant bank of tissue that will act as a valuable resource for the scientific community. For instance, tissue from these animals will be used to further our studies of a 'biomarker' for Alzheimer's Disease, i.e., a method of testing for Alzheimer's Disease early, before symptoms appear.

Who or what will benefit from these outputs, and how?

The data and publications generated from this proposal will have broad short-term benefits to many diverse groups within the academic community and industrial collaborators, especially those seeking to understand the processes underlying cognition and how it can be impaired in health and disease.

The primary application of our new animal model of Alzheimer's Disease, however, is to enable the discovery of more effective treatments that target disease progression, not just symptoms. Therefore, the ultimate long-term beneficiaries of this project are patients with Alzheimer's Disease (along with their families and carers), who would benefit from earlier diagnosis and a companion therapeutic.

How will you look to maximise the outputs of this work?

Publication of peer-reviewed primary research and review articles in high-impact journals; presentations at local meetings (to discuss options to collaborate and share tissue banks), national and international conferences; dissemination of knowledge gained to the public via media or events; collaborate broadly across the Establishment, with other institutions and companies to maximise scope, uptake, and impact, for example, sharing tissue with other researchers (Neuro-Bio, other groups in the Establishment).

We plan to offer the animal model for study and will offer the protocols to researchers or any commercial animal providers/research units.

Species and numbers of animals expected to be used

- Mice: 800

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult mice are a standard animal model in neuroscience research as their brains are close enough to humans to make interpretations about how our own brain works, while remaining as evolutionarily basic as possible. Rodents are the least sentient species that express the complexity of behaviours and symptoms that are observed in clinical populations; existing animal models of neurodegeneration and behavioural data are largely from mice. Furthermore, the anatomical structures implicated in the disease pathology are well described and functionally characterised in mice.

We will also use genetically modified mice that have mutations in genes associated with Alzheimer's disease, which express some of the behavioural and neuropathological phenotypes of the disease, to compare to our new surgical model.

Aged mice will also be used to allow comparison of data from our new model with the effects of normal ageing. Juvenile mice may be used for acclimatisation purposes but will not undergo regulated procedures until adulthood.

Typically, what will be done to an animal used in your project?

Mice will be handled to acclimatise to the experimenter(s) and to any relevant testing environments. In initial experiments, existing genetically modified mouse models of Alzheimer's disease will be further phenotyped for a range of behaviours. These mice will be compared to healthy control mice, as well as aged mice to compare the effects of Alzheimer's-related mutations to natural aging.

In other experiments, healthy mice will be anaesthetised, and a guide cannula implanted into the brain under aseptic conditions. As is standard for human surgery, mice will receive multi-modal analgesia to reduce any post-surgical pain. The new model of Alzheimer's Disease that we would like to create and validate involves infusion into the brain of a compound we believe causes neurodegeneration.

Following at least 1 week recovery from the initial surgery, mice will begin to receive brain infusions (up to 12 over a 6-week period) via an infusion needle inserted into the guide cannula. Although the brain itself contains no pain receptors, and mice cannot feel these infusions, this may be performed under anaesthesia to avoid stress from handling.

Over time, we expect the injections to induce pathology typical of Alzheimer's Disease and associated symptoms. These symptoms will be subtle and only detectable using sensitive behavioural tests. Either concurrently or thereafter, mice may receive infusions of a treatment compound directly into the brain or via intranasal, intra-peritoneal, or sub-cutaneous routes for up to 20-22 weeks.

Mice will be kept in cages in groups or, when scientifically justified, singly. They will have free access to water and, in most studies, food. In one type of study, and for a discrete period of time, mice will have their food intake restricted to approximately 85% of their normal intake. In another, food will have been withdrawn overnight. This is to provide additional motivation in behavioural tests.

Except in pilot studies, all mice will undergo behavioural testing – before and/or after surgery – and experience up to six different tests. These tests will allow us to assess Alzheimer's-like behaviours: levels of anxiety, experience of reward, learning and memory, locomotion, or sleep. Most of these tests are entirely non-aversive and may be considered equivalent to environmental enrichment. Tests will be performed for up to 20-22 weeks after the start of infusions.

Some of the simplest behavioural tests involve placing mice in an open arena and recording their movement; the extent to which mice enter the central areas provides an index of anxiety. Another test measures the value of a reward by associating burrowing material with hidden food reward; the value attached provides an index of mood and motivational state.

At the end of the studies, all mice will be humanely killed.

What are the expected impacts and/or adverse effects for the animals during your project?

We do not expect adverse effects of the neurodegenerative peptides themselves when infused into the brain, just subtle behavioural changes that can only be detected on subtle behavioural paradigms. We expect any post-surgical pain to be controlled using analgesia.

Nevertheless, careful health checks will take place following surgery as well as after each individual brain injection. Subtle Alzheimer's Disease-like symptoms are expected to appear following the start of the brain injection paradigm and initial pilot studies designed to ensure that any impairment in behaviour is not overt and does not disrupt necessary daily behaviours; namely behavioural signs of the disease can only be detected with our sensitive behavioural tests.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Non-recovery: 5%
Mild: 25%
Moderate: 70%

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The detailed mechanistic understanding of causal brain-behaviour can only be studied in a whole organism. Ethically it is not possible to have human participants, so we need to use a mammalian model that is representative of the human. We must also accurately assess the long-term interaction of the molecules that we have developed with intact neurobiological structures and their impact on behaviour which cannot be done in vitro.

Which non-animal alternatives did you consider for use in this project?

Our experiments in mice are informed by, and closely integrated with, complementary non-animal approaches. The development of candidate compounds in our laboratory that trigger, and block, mechanisms underlying neurodegeneration have utilised cultured cell lines and ex vivo approaches. Furthermore, decades of behavioural assessment of patients with Alzheimer's Disease and post- mortem brain tissue analysis has provided sufficient data against which animal models can be evaluated.

Why were they not suitable?

In vitro/ex vivo studies in brain slices can reveal important basic neurophysiological processes, but in vitro results are difficult to link directly to behavioural studies.

Moreover, neuronal interactions within extended structures and between brain structures are difficult, if not impossible, to study using brain slices, as the brain 'slicing' process destroys the relevant neuronal connections.
Cell cultures are a useful tool but do not represent the physiology of intact neural systems, whereas insufficient information exists regarding the neurodegeneration process to establish valid parameters in computer simulations.

Work on the relationship between behaviour and biochemical changes in the brain cannot yet be simulated. Computational models try to capture the phenomena described in extant empirical observations. Ultimately these computer models depend on the data that we produce from animal studies. They cannot therefore precede our studies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Previous studies will be used to estimate the group sizes needed for each experiment. We estimate that approximately 200 animals may be used for behavioural phenotyping of current models and the effects of aging, and 100 animals will be used for piloting the infusion experiments and optimising the dosing protocol.

Given the long duration of the infusion studies, we estimate that an experiment will be started every 6 -12 months over 5 years, including approximately 50 mice (based on power calculations from previous experiments). This will use approximately 500 mice.

We have consulted a statistician regarding how to sufficiently power the study and we will use the NC3Rs Experimental Design Tool to plan individual experiments throughout the project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Pilot experiments will be performed to produce data to perform accurate power calculations to ensure sufficient groups sizes.

Counterbalanced behavioural test batteries will be performed to maximise the data from individual animals and reduce unnecessary duplications of infusion experiments for different behavioural measurements. We will also use the NC3Rs Experimental Design Tool to plan individual experiments throughout the project.

Experiments will be planned with post-mortem tissue analysis in mind to ensure that the maximum data can be obtained from each mouse.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Pilot studies will be used to a) confirm accurate targeting of brain regions, and b) to optimise the brain injection regime to produce a robust model of Alzheimer's Disease that exhibits appropriate behavioural symptoms and brain pathology, whilst not disrupting normal behaviour.

We will also analyse the brain tissue from the mice we use and correlate biochemical measures with behavioural performance to better understand how changes in brain neurochemistry may be associated with altered behaviour.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative

care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mouse models provide an opportunity to better understand disease processes and evaluate treatments; by developing a new, more superior animal model, we also need to compare it to the current best model, which is a genetically altered mouse model.

We will use a range of unconditioned and conditioned, but importantly non-aversive, behavioural tests, including those to assess learning and memory (e.g., T maze, Y maze, novel object recognition, operant conditioning), anxiety-like behaviour (e.g., elevated plus maze, open field), tests for emotionality (e.g., sucrose preference), locomotor assessment and non-invasive sleep monitoring.

Animal welfare is vital for the success of these studies: behavioural tests often require the subjects' inquisitiveness and free exploration; undue stress would therefore interfere with most of our measures. Only the most refined tests have been selected; for example, the Morris Water Maze test was discounted and replaced with a more refined test to reduce any potential distress during swimming. Most of the behavioural tests we will use have been refined by being entirely non-aversive and even considered equivalent to environmental enrichment.

We minimise undue stress of the mice before a test by carefully habituating them to handling using refined techniques such as tunnel handling or cupping (and, where appropriate, acclimatise them to the behavioural environment). Researchers will be trained in the handling technique to reduce variability between individuals. Animals will be handled by the minimum number of researchers to reduce stress from multiple people, and where possible by researchers of the same gender. After handling, mice used in our studies are typically comfortable with the experimenters and start orienting towards the cage entrance and the experimenter's hand when the experimenter approaches. Mice maintain this behaviour throughout our studies, supporting the non-aversive nature of our tests.
In one type of study, and for a discrete period, mice will have their food intake restricted to approximately 85% of their normal intake. In another, food will have been withdrawn overnight (and for a maximum of 16 h). This is to provide additional motivation in behavioural tests. These periods are the minimum period to ensure reliable data acquisition. To accurately monitor sleep, mice will be housed singly, but only for the minimum period necessary. Mice will be culled after this experiment, or re- housed unless not possible due to welfare reasons.

We do not anticipate that these processes will cause any lasting harm to the animals, but careful daily checks will be performed throughout all of our experiments to ensure that the animals are healthy.

Why can't you use animals that are less sentient?

The study of clinically relevant mechanisms underlying neurodegeneration, and the

associated behavioural symptoms, requires a mammalian species and mature animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

- Comprehensive health checks will be performed following surgical procedures and drug administration into the brain to ensure any potential adverse effects (only expected in a minority of cases) are detected as quickly as possible.
- Multi-modal analgesia will be given during surgery to minimise any discomfort during and after recovery.
- Handling of animals to ensure they are comfortable with the experimenter(s) and appropriate acclimatisation to relevant environments will enable behavioural testing to be conducted efficiently and with the minimum of distress to the animals. Many of the behavioural tests being performed are considered to be types of environmental enrichment, due to their cognitively- stimulating nature, and several others involve food rewards.
- Animals will also be monitored and appropriate health checks performed at least weekly once drug administration begins, and during behavioural testing, to identify potential welfare issues early.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The PREPARE guidelines, for the Planning, Research, and Experimental Procedures on Animals: Recommendations for Excellence (norecopa.no/prepare).

The ARRIVE guidelines, used in Animal Research (for the) Reporting of In Vivo Experiments. These are a checklist of recommendations for the full and transparent reporting of research involving animals – maximising the quality and reliability of published research, and enabling others to better scrutinise, evaluate and reproduce it (arriveguidelines.org).

We will also follow the Laboratory Animal Science Association (LASA) Guiding Principles for Preparing for and Undertaking Aseptic Surgery (www.lasa.co.uk/current_publications), and administration of substances will be conducted in accordance with LASA Good Practice Guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will attend local 3Rs events at the Establishment and will attend regular internal welfare meetings. We will also receive email updates and newsletters from the NC3Rs, LASA, and the RSPCA Animals in Science team. We also receive updates from the Named Information and Named Training & Competency Officers regarding opportunities for local seminars and refresher training. Where relevant refinements are identified, the Named Animal Care & Welfare Officer and Named Veterinary Surgeons will be consulted on whether and how these refinements can be incorporated into studies.

50. Mechanisms and therapeutics for brain disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Nucleic acid therapy, Gene therapy, Epilepsy, Brain excitability, Ion channels

Animal types	Life stages
Mice	adult, embryo, neonate, pregnant, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to develop powerful, safe and long-lasting treatments for brain diseases which include epilepsy and chronic pain, and to develop a better understanding of how and why these conditions happen.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This work will give a new level of understanding of how brain activity changes between health and disease, and may lead to urgently needed therapies for people with lifelong brain diseases that cannot be treated with currently available medications.

What outputs do you think you will see at the end of this project?

- A new level of understanding precisely how genes are expressed in our brains and how they interact to give rise to healthy brain function.
- Full preclinical testing of novel genetic therapies which can treat and/or cure brain diseases, such as epilepsy and chronic pain, which cannot be cured with existing medications. This will include an estimated at least five promising therapies for each of chronic pain and epilepsy.
- Better understanding of how epilepsy medications pose neurodevelopmental risks to the foetus when taken during pregnancy.

Who or what will benefit from these outputs, and how?

Short-term: The main benefit will be to fundamental and pre-clinical researchers in neuroscience and molecular biology, as this project will generate a new level of insight into how gene networks shape brain function, and will generate pre-clinical data on new therapies for neurological diseases.

Medium/long-term: This work will provide enormous benefit to people who have currently untreatable neurological diseases, their families and the clinicians who care for them. This benefit will be seen in the longer term as the treatments discovered progress to clinical trials and into clinical use. The most immediate applications of the approaches in this PPL will be to epilepsy (>50 million patients worldwide) and chronic pain (~60 million worldwide). The genetic therapies being developed are designed to provide broad therapeutic benefit to a wide patient-base – regardless of the exact cause in each individual patient – by treating a common pathological mechanism which is over-excitability in the brain.

How will you look to maximise the outputs of this work?

The outputs of this work will be maximised by publishing all findings (positive and negative) in open access scientific journals, in order to disseminate the work as widely as possible within the scientific community. Findings will be presented regularly and openly at scientific conferences and, where appropriate, communicated to patient and public audiences, in order to maximise the reach of the work.

I also work closely with national epilepsy societies, which will maximise dissemination to directly interested individuals and, via these organisations, I regularly interact with people with epilepsy and patient groups. Finally, I am embedded within the local and regional epilepsy research networks comprising scientists and NHS clinicians. These clinical collaborations further increase the reach and relevance of all outputs generated within the project.

Species and numbers of animals expected to be used

- Mice: 1095

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project will only use mice. Mice are an ideal model organism for epilepsy studies because their brain is a close approximation to humans, and many valid and reproducible models are available. The reproducibility means that smaller animal numbers are needed for statistical analyses, and the proximity to human brain supports predictive validity and translational potential of the work.

Furthermore, mice are highly amenable to genetic manipulation, creating models of genetic brain diseases. For these reasons, mice are generally considered to be the gold-standard in epilepsy research. We will use adult mice for epilepsy studies as we are aiming to treat adult epilepsy.

For chronic pain study, we will use a well-established mouse model in which capsaicin in injected into one of the animal's paws to induce local inflammatory chronic pain following, without the need for invasive surgery or more severe models.

This model is appropriate because it recapitulates key features of human chronic pain, including over-activity in the brain, which we are aiming to treat with our novel therapies.

To study prenatal epilepsy medication exposure, epilepsy medications can be easily administered to pregnant mice without harm or distress, and then possible harm to the embryo and associated mechanisms can be studied in their offspring. Mice provide an ideal model because their relatively short gestation period and relatively large litter size means that data can be generated quickly and in a reproducible manner. The use of inbred mice means that any effects that we observe will take place in the context of an identical genetic background, thereby minimising the variability of the approach.

Typically, what will be done to an animal used in your project?

In general, mice may (or may not) undergo a chronic neurological disease model (either epilepsy or chronic pain) and will then be treated with an experimental genetic therapy which aims to reduce the disease severity. Epilepsy models usually involve an aseptic surgical intervention to inject an epilepsy- causing chemical directly into the brain, whereas the chronic pain model in this project can be triggered by injecting a substance into one of the mouse's paws. The genetic therapies will all be delivered via aseptic surgical injection into the brain, which is currently the only well-characterised and reliable method to deliver them. In the case of epileptic mice, we will mount a cannula into the skull during the first surgery (where epilepsy is induced) so that the second surgery (genetic therapy) can be brief and does not require re-opening any wounds. In most cases, the epilepsy/pain will be allowed to develop for roughly 1 week to understand how the model presents in each mouse, and then the therapy will be administered. Gene therapies (which use harmless viruses to introduce DNA into the brain) will be allowed to express for up to 3 weeks during which time their impact on disease progression will be monitored. Antisense oligonucleotides (short DNA sequences which bind directly to other genetic material in the brain) typically act much faster, and their impact on disease will be monitored for up to 2 weeks after delivery. Some mice will also undergo behavioural testing.

These tests are do not cause any suffering and assess the natural behaviours of rodents including seeking food and exploring novel objects.

What are the expected impacts and/or adverse effects for the animals during your project?

- Mice may experience transient weight loss after aseptic surgery, although this typically only persists for 1-2 days and is mitigated by provision of wet food.
- Possible pain associated with aseptic surgical procedures will be manages using analgesics.
- Mice may experience minor irritation of wounds for 3 days post-surgery, and any animals experiencing this will receive appropriate analgesia.
- A small percentage of mice will experience re-opening of surgical wounds within 3 days of surgery, which will be repaired a maximum of one time.
- Epileptic mice may experience additional distress associated with the development of epilepsy and seizures. This is an unavoidable aspect of the disease which we are modelling. Mice will be monitored daily for any signs of pain/distress, and handling limited to avoid stress.
- Mice used for pain studies will experience moderate chronic pain, for a maximum limit of two weeks, as this is the clinical phenotype that we want to model and treat.

The total time between initiating chronic pain, delivering a genetic therapy, and completing the experiment will be limited to two weeks.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice will experience different severities depending on what protocol they undergo.

Non-recovery – ~10% of mice will be euthanised under general anaesthesia in order to perform a specialised methodology to dissect viable brain tissue for electrical recordings. These mice will never be conscious during or following any procedure.

Mild (~5% of mice) – in the protocol to study prenatal medication exposure, the pregnant female mice will be treated chronically with antiseizure medications. These are known to have a risk of mild cognitive impacts, but are not expected to cause lasting harm or distress.

Moderate (~85% of mice) - due to aseptic surgical procedures and the use of chronic disease models which can feature limited levels of pain/distress over a period of two weeks (chronic pain) or spontaneous recurrent seizures (chronic epilepsy). The most common adverse effects arise due to recovery from stereotaxic surgery. This can include a small temporary loss in body weight, or irritation as surgical wounds heal.

Such models are commonly used in the research field and as such are continuously refined to minimise adverse effects as much as possible. At the end the animals will be humanely killed and then their brains dissected for further examination. This increases the

information gained from each animal and also negates the need for more invasive in vivo recordings.

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We want to understand the mechanisms controlling the excitability of our brains, how these change in epilepsy and chronic pain, and how we can restore them to treat these diseases. Brains are highly complex systems made up of large numbers a different types of cells, each of which contains a huge number of different genes and which are intricately interconnected. Therefore the manipulation of individual gene(s) in individual cell types will have emergent effects on the entire brain system which cannot be captured in any other type of model system.

Which non-animal alternatives did you consider for use in this project?

- Dissociated cell cultures
- Human-derived stem cell culture
- Organ on a chip (brain cell cultures grown directly onto a high density grid of electrodes)
- Long-term brain slice cultures
- Computational models
- Human brain tissue from epilepsy surgeries

Why were they not suitable?

- Dissociated and human stem cell cultures do not form physiologically realistic brain networks and therefore do not capture the complexity of the brain beyond the level of individual cells. They can however be used to measure effects at the single cell level and will be used as a replacement for certain scientific questions where this approach is suitable. Organ-on-a-chip is a similar approach in which cultures are seeded directly onto a dense grid of electrodes. This can give useful information about network interactions between all of the cells in the cultures, with the same substantial caveat that the cells do not form realistic networks which recapitulate those in the real brain.
- Brain slice cultures only represent isolated brain circuitry and they flatten and develop abnormal neural connections over time in culture, and ultimately generate spontaneous seizures themselves. They therefore lose realism over time and also complicate any scientific findings because the health of the brain slice itself is highly variable and changes over time. Slice cultures may be used as a replacement in specific cases where we want to express a genetic therapy in isolated tissue.
- Computational models are typically highly limited and capture brain cells/networks at a

highly reduced level of complexity in order to make them computationally viable. In silico approaches such as the modelling of genetic networks will be used to generate hypotheses for animal work, but are unlikely to serve as a suitable replacement for any experiments within this PPL.
- Epileptic human tissue is difficult to obtain, and is often unhealthy and highly variable. In the case of testing genetic therapies for epilepsy, we will likely use this type of model in parallel with animal work to enhance the potential to move our strategies to clinical development.

In general, these models will be used to screen potential treatments and to generate hypotheses for in vivo work. Therefore, even though they cannot replace in vivo experiments, they can aid in reduction and refinement.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

In general, sample sizes are based on my own previous experience of using the relevant in vivo models, and published datasets were used to estimate relevant effect sizes and variability of datasets. From existing data, we know that mice undergoing our epilepsy model will experience an average of roughly 15 seizures per day, with each seizure lasting for roughly between 5-30 seconds. Mice typically lose consciousness during these seizures and so do not suffer during the seizure. Where possible, shared control groups have been used (i.e. comparing multiple treatment groups to the same vehicle control group) in order to reduce animal use. Studies will be blinded throughout experimentation and analysis, and animals will typically be assigned to treatment groups using a randomised block design. We will use a mixture of male and female animals and factorial experimental design to rule out any effect of sex on our experiments, without the need for the use of additional animals.

Data output from each animal will be maximised through the use of ex vivo brain tissues, following in vivo work, for further biophysical/molecular/anatomical analyses.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

- Use of a specific statistical design which allows us to assess sex differences between treatments without using extra animals.
- Use of shared control groups where possible.
- Use of in silico/in vitro models to generate hypotheses for in vivo work.
- Use of an experimental design where tests are repeated over time in order to account for environmental changes which might impact the experiments.
- Use of a highly reproducible epilepsy model to reduce variability, and followed published epilepsy- specific guidelines from the NC3Rs.
- Standardised husbandry procedures to minimise environmental variability.

- Closely followed ARRIVE2.0 guidelines to ensure that data generated can be reported to a high standard.
- Followed PREPARE guidelines in order to plan research experiments to a high standard.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use pilot studies to test novel genetic interventions and generate data for power calculations.

We will collect ex vivo brain tissue from all animals which will generate further mechanistic data about our genetic manipulations. This means that we do not need to carry out more invasive procedures in vivo in order to gather similar information.

In instances where it is scientifically valid, we will use non-animal alternatives such as cell lines to test and/or verify particular biological effects at the level of individual cells.

Our work will be supported by computational models and database searches which yield the most promising therapeutic targets for epilepsy, thereby prioritising interventions which have the greatest chance of success and minimising the aspect of trial and error.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

- Modelling epilepsy in mice using chemicals injected into the brain. The direct injection of these substances into the brain means that they act upon a very specific part of the brain and don't have any impact on the rest of the body, therefore minimising their effects. In general, the focal models used involve less frequent seizures than genetic models.
- Acute seizure models, induced by either systemic kainic acid or systemic pentylenetetrazole. These models provide a quick readout of seizure susceptibility without the need for a more prolonged chronic model. In this case, the models are more refined because they are short-lasting (maximum one hour duration). They cannot completely replace chronic models because they do not capture the epileptic brain state, but they do provide information on efficacy without the use of longer-term models.
- Induction of chronic pain using capsaicin. The direct injection into the paw is a refinement over many other chronic pain models because it does not require surgical intervention to initiate pain, and therefore negates the requirement for an extra surgical procedure. The use of capsaicin rather than other agents (e.g. complete Freund's

adjuvant) is a refinement as it triggers a more limited acute pain, whilst still capturing the necessary disease mechanisms that we aim to study and treat.

Why can’t you use animals that are less sentient?

It is critical to epilepsy and pain in a developed adult brain to capture the full pathophysiology of the disease.

Embryonic life stages would not be appropriate for this work because the brain is at a very different developmental stage and treatments would not have the same effect.

We use mice because their brain is broadly similar in structure, function and genetics to humans. Other animals would not capture human brain physiology well enough to give to able to test new epilepsy and pain therapies accurately.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Daily welfare monitoring using standardised objective scoresheets with appropriate humane endpoints.

The use of standardised and well established aseptic surgical procedures and models will minimise harms.

Post-operative care will follow NVS and NACWO advice to minimise harms.

Refinements can include use of gel food to add weight post-surgery, appropriate analgesics in consultation with NVS, and group housing post-surgery.

Behavioural tests are not anticipated to be harmful, although mice will be acclimatised to any novel environment to minimise anxiety during tests.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Opportunities for improving animal welfare in rodent models of epilepsy and seizures (J Neuroscience) - This publication arose from an expert-led working group and gives epilepsy-specific recommendations for refinements which include: Choice of animal model; Induction procedures; In vivo recordings; Perioperative care; Welfare assessment; Humane endpoints; Social housing; Environmental enrichment;

Reporting and data sharing.

PREPARE guidelines to ensure that experiments are planned to a high standard.

The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research (PLOS Biology) - updated ARRIVE guidelines on the reporting of animal work to maximise reproducibility.

Unilateral hippocampal CA3-predominant damage and short latency epileptogenesis after intra- amygdala microinjection of kainic acid in mice (Brain Research) - Original publication

describing the animal model to be used.

The intra-amygdala kainic acid model has been refined through experience in my previous two lab groups.

LASA guidelines for administration of substances and aseptic surgery.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Working closely with the NACWO, NIO, local 3Rs manager, and AWERB throughout the project to continuously explore possible refinements to our models.

Following any updated guidance that arrives via the NC3Rs or similar organisations.

Engaging with NC3Rs at conferences and workshops.

51. Understanding platelet function as a central regulator of cardiovascular function and development

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph

Key words

Platelet, Thrombosis, Cell signalling, Cardiovascular risk factors, Haemostasis

Animal types	Life stages
Zebra fish (Danio rerio)	adult, neonate, juvenile, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand how the regulation of platelet function contributes to cardiovascular disease and function. Using this understanding we aim to identify more effective strategies to prevent heart attacks, strokes and other conditions in which platelets are implicated.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Blood platelets are small cells in the blood which, when activated, play an important role in the prevention of excessive bleeding at sites of injury. This process is called haemostasis. Conversely, inappropriate platelet activation can lead to heart attack or stroke and contributes to an estimated 40% of cardiovascular deaths. This process is called thrombosis. It is the role of platelets in thrombosis that has led to the generation of many medicines aimed at reducing platelet activation with new treatment strategies for certain patient groups such as the elderly and patients with cancer, obesity and diabetes being developed. It is unclear why certain patient groups are at a greater risk of developing cardiovascular disease or why a third of patients go on to have a second heart attack or stroke despite anti-platelet therapy.

In addition to their role in thrombosis and haemostasis, platelets are now recognised to play a role in other normal and disease processes. These roles largely result from the communication of platelets with other cells, for example with cancer cells during metastasis, immune cells during inflammation and lymphatic endothelial cells, the cells that line the body's lymphatic system, during the formation of new lymphatic vessels, a process known as lymphangiogenesis. However, the interaction of platelets with other cells in the body may be an important factor for the development of cardiovascular disease in certain patient groups. The mechanisms involved in these processes are unclear but some of the pathways are shared with those involved in haemostasis and thrombosis.

What outputs do you think you will see at the end of this project?

At the end of the project we expect to have:

New understanding of how platelet function contributes to cardiovascular health and disease.

New understanding of how risk factors contribute to cardiovascular health and disease.

Use this new knowledge to determine which cellular processes may be effectively therapeutically targeted.

Disseminate the results of our research through the publication of our research in peer reviewed journal articles and at conference talks.

Who or what will benefit from these outputs, and how?

The longer term benefits of this project will be to human health. Cardiovascular disease leads to significant poor health and death globally. In 2022, 39,000 people in England died prematurely from cardiovascular conditions (BHF). These cardiovascular conditions have platelets as a contributing or causative factor. It is not clear why, but the number of people dying from cardiovascular disease has started to increase. This may be in part due to an increasingly unhealthy population, with rates of obesity and metabolic disorders also increasing. Outputs from our research will lay the groundwork for translational research and may lead to the refinement of current therapies and the development of new ones.

How will you look to maximise the outputs of this work?

We will publicise our findings to the broader scientific community, industry and the wider public through peer reviewed research publications, conference presentations and engagement activities. This will maximise the contribution and translation of our work for human benefit. Additionally, by publicising our research findings at conferences and meetings new collaborations will be developed, furthering innovative research knowledge and opportunities.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 5600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

There are currently no alternative approaches that fully model the human cardiovascular system. To understand the role that platelets play in the complex process of cardiovascular health and disease animal models are used to understand the roles of specific proteins and processes. The aim is to identify proteins or processes that can be therapeutically targeted to prevent or treat cardiovascular disease and complications that arise from cardiovascular disease such as heart attack or stroke and to understand how risk factors, such as high calorie diets and plastic pollution, impact platelet function.

Before the start of any research using animals, work using human platelets taken from blood donors, or cell lines *in vitro*, will be carried out.

Zebrafish are used to identify and understand genes which affect human development and diseases as many genes and cell biological processes are found in both humans and zebrafish. This similarity is shared between humans and zebrafish with regard to platelet function and many of the known platelet processes are common to both. The ability to directly manipulate the Zebrafish genome is used by researchers to enable human disease to be modelled and for the contribution of cellular processes investigated. Combined with the transparency of the Zebrafish embryo, approaches which are not possible with mouse models can be adopted and used to understand how platelets contribute to thrombosis, haemostasis and other processes that platelets play a role. Zebrafish can also express fluorescent proteins that allow molecules, cells or tissues to be visualised in real time and *in vivo*.

These can be used to understand how platelets contribute to thrombosis, haemostasis and other processes that platelets play a role.

Typically, what will be done to an animal used in your project?

Typically, fish that have been genetically altered will be allowed to breed via natural spawning. These fish will lack specific proteins, will express a mutated form of the protein or will express fluorescent proteins that allow cells and tissues to be visualised. This will

allow us to understand the role specific proteins play during platelet function and the fish will typically not display any abnormal behaviour that may indicate poor health.

It may be important to know the genetic makeup of a fish. Fish that express fluorescent proteins will be identified using non-invasive microscopy. Occasionally DNA is needed to determine the genetic make-up of a fish. Typically, the DNA will be extracted from non-invasive mucus swabs from anesthetised fish or by using non-invasive technology when the fish are in a larval life form.

Occasionally, for example when non-invasive approaches are not successful due to poor recovery of DNA, a small section of fin may need to be removed to enable the genetic makeup of the fish to be determined. The removal of the small section of fin will take place while the fish is anesthetised.

Anaesthesia is given to the fish in their water and following the procedure the fish will be monitored while the fish recovers from the anaesthesia.

To understand the impact of a high calorie diet on platelet function and the processes that platelets are involved in, fish will be fed a high calorie diet to induce obesity and changes in metabolism. The fish will display normal feeding behaviour and will be carefully monitored while on this diet. To understand the impact of diet or the role specific proteins play during platelet function fish may undergo one of the following procedures which are performed under anaesthesia without recovery; the thrombosis assay, the haemostasis assay or the blood taking procedure. Fish will not experience any pain during these procedures. To understand the impact of a parental high calorie diet, fish will be allowed to breed naturally and the progeny will be fed a high calorie diet to induce obesity and changes in metabolism.

To understand how proteins play a role in thrombosis and haemostasis fish may undergo one of the following procedures; the thrombosis assay, the haemostasis assay or the blood taking procedure. These procedures will be performed under anaesthesia without recovery. Fish will not experience any pain during these procedures.

For the thrombosis and haemostasis assays, injury can be induced in a number of different ways, such as the application of chemicals, the use of a laser or by physically damaging a blood vessel. These trigger the formation of a thrombus which can be observed using microscopy. The response to injury can be used to understand how different pharmacological treatments may impact the formation of the thrombus. It may also be necessary to remove blood from fish to investigate platelet function. This procedure is performed under anaesthesia without recovery.

What are the expected impacts and/or adverse effects for the animals during your project?

In most cases the breeding of genetically modified zebrafish will result in zebrafish that do not display any abnormal behaviour that is indicative of ill-health.

Some fish with mutations which impact haemostasis may display bleeding in the form of blood spots under the skin and some fish with mutations which impact the vascular development may display oedema, ascites and/or pericardial effusion. Fish will be carefully monitored and will be humanely killed before 5 days post fertilisation (dpf) or at

the onset of clinical signs (the development of significant haemorrhage, greater than blood spots, or severe oedema, ascites and/or pericardial effusion). In fish, haemorrhage can present as pale gills, abnormal swimming behaviour and signs of bleeding around the eyes, fins and skin.

Potential adverse effects will be discussed with staff before work commences. This will also include a training pack with reference images assembled with the support of the Named Veterinary Surgeon and aquatics expert.

The fish on the high calorie diet may gain weight compared to fish maintained on a normal diet. The progeny of fish fed a high calorie diet may also gain weight compared to fish maintained on a normal diet.

The thrombosis, haemostasis and blood taking procedures will be performed under anaesthesia without recovery. These fish will not experience any pain during these procedures.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Zebrafish: >60% subthreshold <20% mild <20% moderate.

What will happen to animals used in this project?

- Killed
- Kept alive at a licensed establishment for non-regulated purposes or possible reuse

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In order to understand how proteins play a role in platelet function it is necessary to generate modified platelets which either lack the protein or express an appropriate mutant form. Platelets however do not have a nucleus and therefore cannot be directly genetically manipulated like nucleated cells. To achieve this they need to be modified at the level of the precursor cell, the megakaryocyte. Despite ongoing efforts to generate platelets *in vitro* from megakaryocytes and induced pluripotent stem (iPS) cells there are currently no accessible and reliable *in vitro* methods that generate platelets in sufficient numbers for functional studies that have retained hallmarks of normal platelet behaviour when compared to freshly purified human platelets. Taken together there are significant limitations of using *in vitro* platelet production to enable an understanding of the molecular mechanisms governing platelet function to be achieved. The emergence and development of this technology in the literature will continue to be monitored and adopted where possible.

In addition to the lack of a nucleus in platelets, the complexities involved in the interaction of platelets with other cells and the extracellular matrix environment *in vivo* cannot currently be fully modelled *in vitro*. The impact of diet involves multiple bodily processes which cannot currently be modelled *in vitro*. This project seeks to use the Zebrafish as a model to study and further our understanding of platelet biology in the *in vivo* environment. The majority of the work will be performed in the larval form before the onset of independent feeding.

Which non-animal alternatives did you consider for use in this project?

Where possible research will involve the use of *in vitro* systems such as cell culture and human tissue assays to complement any animal work. Where candidate genes can be identified from previous work, preliminary work will be performed *in vitro*, for example, to confirm that appropriate proteins in zebrafish have been identified, particularly with regard to protein interacting partners. Ongoing work will continue to develop, characterise and standardise alternative approaches to using animal models such as 'vessel on a chip' technology.

Why were they not suitable?

In addition to the lack of a nucleus in platelets, the complexities involved in the interactions of platelets with other cells and the extracellular matrix environment *in vivo* cannot currently be fully modelled *in vitro*. Many of the processes involved in the development of cardiovascular disease and its complications involve multiple bodily processes, beyond the immediate blood vessel environment.

This complexity cannot yet be modelled *in vitro*. The emergence and development of technology in the literature to address these problems will continue to be monitored and adopted where possible.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

In some cases a pilot study may be appropriate to refine the estimate and to support methodological decisions.

Experiments will be designed to minimise the number of fish needed.

We have estimated that we will require 4000 breeding fish to generate larvae. Older progeny may be used as replacement breeding stock and in experiments (for example fish will be used to determine how risk factors (for example obesity and proteins that impact thrombosis, haemostasis and processes that platelet play a role (1500 fish)).

Gamete expression is needed to preserve fertility and preserving lines (100 fish). The preservation of lines will prevent keeping living stocks unnecessarily.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To identify scientifically important responses the sample size estimation for our planned experiments will be calculated using software. We will assume that the results will be normally distributed, and where available, biologically important differences will be identified for the essential outcomes of the research question from the literature. To potentially minimise the number of animals used we will use a group-sequential design where possible, with interim analyses pre-planned into the study design.

Where published research is not available, pilot studies will be performed to determine treatment effects and variance estimates for sample size calculations.

Sample size estimates will be calculated using a significance level of 0.05 and power of 90%. The NC3Rs Experimental Design Assistant will support the experimental design process.

For the generation of new lines, where possible we will identify carriers/founders before the onset of independent feeding. When this is not possible we will raise approximately one hundred embryos to adulthood to identify a minimum working stock of positive carriers/founders. Through collaboration and monitoring of the literature we will also identify developing techniques that increase transgene identification and incorporation leading to a reduction in the number of transgenic animals generated unnecessarily. For example the use of multiple targeting CRISPR guide RNAs in to the Zebrafish embryo can determine if a particular approach necessitates the generation of a particular transgenic line early on.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Before the start of any experiment an experimental plan will be drawn up. This is important for the transparency and reproducibility of our research. The plan will include clearly defined hypotheses, objectives, experimental data and analysis. The NC3Rs Experimental Design Assistant will be used to support the experimental plan including the determination of the number of experimental groups. To potentially minimise the number of animals used we will use a group-sequential design where possible, with interim analyses pre-planned into the study design. Where previous literature is not available a pilot study will be performed to determine treatment effects and variance estimates for sample size calculations.

A Zebrafish Embryonic Genotyper (ZEG) enables rapid automated DNA extraction of live Zebrafish larvae. This method will determine if a particular approach necessitates the generation of a transgenic line early on and removes the need for fish to be grown to adulthood (2-3 months) before genotyping by mucus swabs or occasionally by fin clipping.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The fish will be housed in tanks covered by a service contract and a room with appropriate dawn/dusk automatic lighting. The tanks continuously circulate water and maintain temperature, pH and salinity with an automatic monitoring and alert system. Enrichment in the form of live feed may be given.

Evidence suggests that zebrafish have a preference for a gravel like tank bottom, therefore enrichment with gravel effect tank runners may be used.

The majority of studies will be performed in larvae before the onset of independent feeding. The methods for generating mutants and transgenics will be optimised to minimise the number of animals used to ensure that adverse effects from this method are minimal. These approaches will also be supplemented with the use of morpholino knockdown which is effective in the early stages of development before the onset of independent feeding.

Where possible, the administration of substances or control substances that modulate platelet function alone or in combination will be by the tank water. We will aim, wherever possible, to adjust pH to avoid any undue distress for the fish and match normal environments. The administration of substances or control substances to the tank water will be the preferred route. Physical routes of administration will only be used if necessary.

Pilot experiments will be performed to determine appropriate dosing and to identity any potential adverse effects. The thrombosis, haemostasis and blood collection assays will be performed under terminal anaesthesia.

Why can't you use animals that are less sentient?

The majority of studies will be performed in larvae before the onset of independent feeding. Thrombosis, haemostasis and blood collection procedures will use fish that are anaesthetised without recovery.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our approaches will continue to be refined by monitoring the literature and through communications from colleagues and organisations such as the NC3Rs. An example of a refinement/replacement approach was the acquisition of a Zebrafish Embryonic Genotyper (ZEG) to enable rapid automated DNA extraction of live Zebrafish larvae. This may remove the need for the fish to be reared to an adult or the need to sample DNA in adults.

Occasionally DNA is needed to assess the genetic makeup of a fish. The preferred route

will be for DNA to be extracted from non-invasive mucus swabs from anesthetised fish or by using non-invasive technology when the fish are in a larval life form. Occasionally a small section of fin may need to be removed to enable the genetic makeup of the fish to be determined, for example when mucus swabs do not work.

Refinements to housing will continue, for example evidence suggests that zebrafish have a preference for a gravel like tank bottom, therefore enrichment may be provided with gravel effect tank runners.

Sand effect tank runners may also be used during breeding as a refinement strategy. Live food may also provide additional enrichment for the fish.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The NC3Rs website will be used by the group as a source of information, to keep updated on best practice and guidance on experimental design, including their Experimental Design Assistant. The group will also refer to the PREPARE guidelines in the preparation of new experiments. The group will use the ARRIVE guidelines to ensure transparent and reproducible reporting of our work for publication. Collectively the group will monitor the published literature for refinement approaches.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

In addition to the published literature, external communication mechanisms will be maintained with training organisations, other licensed establishments, the Home Office, and relevant external organisations to monitor developments. Communication with external colleagues through named persons, discussion groups and attendance at accredited meetings are all sources of information which could help define strategies of refinement. Attendance at 3Rs seminars, culture of care events and networking meetings will also inform the development and implementation of 3Rs approaches for all members of the group. Group members will also have training in experimental design, and the PREPARE and ARRIVE guidelines.

The NC3Rs website and circulated newsletters from our Named Information Officer (NIO) will be monitored for information and training associated with research involving fish. Guidance from our Named Veterinary Surgeon (NVS) will also be sought regarding the implementation of the 3Rs.

52. Developing fertility control as a management tool for grey squirrels

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Fertility control, Grey squirrels

Animal types	Life stages
Rats	adult, pregnant
Grey squirrels	juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to produce an effective fertility control system suitable for use on grey squirrels in the field.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The grey squirrel has become widespread across much of England and Wales and is continuing its expansion northward into Scotland [Mammal Society, 2018].

Consequently, the degree and extent of the problems caused by grey squirrels have increased, which include threats to forestry and biodiversity. Importantly the continued spread of the grey squirrel also threatens the remnant populations of the endangered red squirrel where they still occur across GB.

The only legal and effective tools to manage populations of grey squirrels are lethal methods (shooting and live trapping with dispatch). Both are inefficient (especially at low densities of squirrels) and unsuitable in many areas where grey squirrels are common (e.g. urban and sub-urban landscapes).

Modelling work has indicated the potential value of fertility control in helping manage problems caused by grey squirrels when integrated into a coordinated landscape-scale control program alongside other (lethal) methods. Fertility control may permit the cost-effective control of squirrel populations at low- densities, as well as the management of populations where lethal methods are socially unacceptable (e.g. urban areas) or technically unfeasible. The most practicable approach to delivering fertility control for grey squirrels is using oral contraceptive delivered in baits to free-ranging squirrels. However this requires detailed studies to:

- identify and characterize fertility control agents which are suitable for squirrels (e.g. safe and with promising properties for fertility control)
- develop and optimize the most practicable and effective formulation for a self-dosing deployment. For example the oral delivery of an agent may include research into the optimum combination of other ingredients such as adjuvants, additives, baits, preservatives etc.
- validate the efficacy and ensure the humaneness of the most promising contraceptive in a proof of concept experiment
- quantify key aspects of grey squirrel behaviour and ecology in the field to support the design and deployment of the field-based devices used to administer the contraceptive
- underpin mathematical models which will be used to provide replacement and reduction for this program of work
- enhance the benefits of the research by demonstrating the utility of the work to a wider audience and practitioners

What outputs do you think you will see at the end of this project?

We will have developed formulation(s) with the necessary properties to contribute to an effective, humane and practicable fertility control system and selected the best candidate contraceptive for a proof of concept experiment using a breeding colony of grey squirrels maintained for the purpose.

We will have undertaken captive experiments demonstrating the efficacy and humaneness of one or more formulations.

We will have developed devices and deployment strategies suitable for use with the most effective and practicable contraceptive.

This project intends to achieve a oral fertility control proof-of-concept in rats initially, as a model species, and subsequently a proof-of-concept in squirrels that could be taken forward as a product that could be considered for registration and use in a fertility control

system for this species.

Who or what will benefit from these outputs, and how?

If successful we will have developed a system comprised of tools (fertility control agent, formulation, delivery device and deployment strategy informed through mathematical modelling) which can be adopted by practitioners in the field and used as part of a comprehensive grey squirrel control strategy. This should bring benefits to the economy (protecting crop trees), biodiversity (where trees and birds are threatened by grey squirrels), as well as supporting the conservation of the red squirrel. This work also acts as the foundation stone upon which further development of any of these tools may produce enhanced benefits in the future (e.g. application to other species and the solution to other human- wildlife conflicts).

Even if only partially successful, we will have driven forward the development of science in one or more of these component tools and contributed to a number of areas of basic science. Particularly the understanding of the reproductive physiology of grey squirrels, their ecology and behaviour in the field, as well as some of the basic principles of developing immunologic approaches for wildlife applications which may bring benefits in other areas or for other species.

How will you look to maximise the outputs of this work?

Elements of the work will be published in the peer-reviewed literature and will be disseminated at technical and scientific conferences as well as meetings of practitioners and stakeholders.

The major funding body associated with the program of work (a consortium of charities and stakeholders as well as the government) are both keen to see any benefits accruing from this work translated into practical benefits as quickly as possible, and communications plans are already being discussed.

Species and numbers of animals expected to be used

- Rats: 250
- Grey Squirrels: 820

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Only the study of grey squirrels allows us to develop and demonstrate an effective and benign self- dosing fertility control system intended to reduce their populations in the field.

The behaviour of free living squirrels can only be studied in the field. Some animals will also be brought into captivity to permit the robust science and inference necessary to develop and test our contraceptives.

Especially in the initial evaluation of fertility control agents or formulations, laboratory rats will be used as a model species as they represent an effective replacement and one more suited to the laboratory environment and their reproductive cycle being shorter means that the studies can be quicker.

Typically, what will be done to an animal used in your project?

Grey squirrels will be used in two types of study. In the field animals will be caught and marked using PIT tags and/or fitted with non-invasive tracking devices (e.g. collars). We may use anaesthesia to make sure that this work is as practical as possible. These animals will be released back into their natural environment. Animals fitted with tracking devices may be recaptured for device removal/replacement as necessary. Some squirrels will be brought into a specialist wildlife facility in the laboratory to participate in experiments to develop a contraceptive. This facility is set up to care for the squirrels and meet their welfare needs. Typically these animals will be administered with a fertility control agent or formulation, and their responses measured using blood samples or non-invasive techniques (e.g. ultrasound) which require the squirrels to be handled (refined using anaesthesia).

Dosing squirrels may involve injections or other regulated methods but candidate contraceptives are expected to often be self-administered in food or drink.

Rat (used as a model species) will be administered with a fertility control agent or formulation, and their responses measured using blood samples. This will involve injections, other regulated methods (gavage or oropharyngeal dosing) or be self-administered in food or drink.

What are the expected impacts and/or adverse effects for the animals during your project?

As our goal is the production of a fertility control system, suitable for use in the field, free of harmful side effects to the squirrels, or harmful effects on the environment.

Most of the laboratory based research we will undertake will be using substances which are not intended to produce any harms to squirrels, and are intended only to reduce their reproductive capacity. Developing the contraceptive will require some squirrels to be injected. More generally, many squirrels will need to provide periodic blood samples or be handled and observed closely (e.g. ultrasound examinations).

These are best done by anaesthetising the animal, which reduces the stress it experiences as well as permitting work to proceed calmly and efficiently to produce the very best science. A small number of animals might react poorly to some treatments but close observation and rapid interventions to limit harms will ensure that suffering is minor and brief.

Some rats may be injected with a fertility control agent where they may suffer some mild and transient pain that is not expected to last more than 72 hours. There may be an injection site reaction manifesting as sterile granulomas but causing no pain to the rats. Rats administered the fertility control agent via oropharyngeal dosing may experience some mild respiratory distress if the agent is viscous although this should be overcome by

splitting the treatment into sub doses.

The grey squirrels we study in the field should experience no significant adverse effects.

Indeed, competent staff will undertake a number of checks to ensure that the animal are fit for release, free of unintended injury, and able to continue living in the wild without detriment.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most animals (estimated at 90%) will experience a mild severity. No animal will exceed a moderate severity.

What will happen to animals used in this project?

- Killed
- Set free

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Developing a contraceptive for grey squirrels and a delivery device for its use in the field, as well as a strategy for deploying it to manage the environmental and economic impact by grey squirrels cannot be done without research using animals.

Whilst research discovering and describing new agents which can produce fertility control in squirrels can use other rodent species as models for some aspects of the work (i.e. laboratory rats), key experiments still require us to study grey squirrels to check and confirm our research. Using grey squirrels is essential to demonstrate that our contraceptive is safe for grey squirrels and effective. In addition, the practicability and cost-effectiveness of the system also depends on the performance of the devices we develop and how wild free-living squirrels behave around them, requiring the study of wild grey squirrels in the field.

However, computer modelling study is running in parallel with the animal study to refine parts of the field trials.

Which non-animal alternatives did you consider for use in this project?

Grey squirrels are an unusual study species. Not much is known of their reproductive physiology and less is known of how to prevent their reproduction. As such non-animal methods known to robustly replicate their reproductive physiology are not available;

requiring the use of animals to undertake the basic science as well as research how to control their fertility.

However, we do use mathematical modelling to identify when experimental work with animals has produced a contraceptive sufficiently good to use in the field. The modelling takes the empirical estimates we produce as part of this study (both of reproductive inhibition in captivity and behaviour in the field) and combines these to predict the efficiency of our fertility control system at the population scale across realistic simulations of real-world landscapes. This avoids undertaking extensive and lengthy study of animals in the field to provide the same evidence.

Why were they not suitable?

There are no non-animal alternatives to study the details of the reproductive physiology of grey squirrels, its inhibition using previously unidentified contraceptives, or describe for the first time the behaviour and ecology of grey squirrels in the field when presented with our self-dosing devices.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have used our previous experience of identifying and characterising fertility control agents or contraceptives in other species to estimate how many animals we might use to achieve our goal of producing a fertility control system for grey squirrels.

Where possible the most efficient statistical designs will be used (e.g. factorial designs), though where the constraints of animal housing (confounded blocking) or the measurement of unwanted interactions groups. Excepting specific study types (e.g pilot studies) many experiments will follow a pattern comparing a number of treatment groups with a control and use around 30 animals.

Laboratory rats are useful for studying some of the basic science around squirrel reproduction and fertility control, or the initial work in describing the potential for a new substance or its safety to squirrels. Whilst we expect most studies to be relatively small (use around 30 animals) we cannot know how many studies we may need to achieve our aims and have specified a number similar to that used on previous similar licences.

However, we will always need to check our results in experiments using squirrels to make sure that our understanding is sound and expect to use a similar number of squirrels (250 animals).

Our plan includes a proof of concept study where we demonstrate the safety and efficacy of our chosen contraceptive, and this needs to use the squirrels in our breeding colony.

Our breeding colony is currently established at 80 grey squirrels. It is likely that some animals will be lost to old age through the course of study and need replacing. Though this number is unlikely to be large it is difficult in this application to estimate accurately how many additional animals may be needed to maintain the colony in anticipation of our final proof of concept study. We have estimated the number needed here will not exceed 150 squirrels. An additional 25 animals might be needed to just produce the basic descriptions of squirrel reproductive physiology which we think will help us to produce better science as well as help us to design shorter and more refined studies.

Pilot studies will be undertaken in a controlled environment to assess and confirm that tracking collars do not cause any harm to the squirrels when attached. It is estimated that up to 20 squirrels will be used for this purpose.

About half of the grey squirrels we might use will be animals which have marked with a PIT tag and/or fitted with a tracking device such as a GPS collar then released back into the wild to continue their life there (approximately 250 squirrels). The number of squirrels we actually use in field experiments may be much smaller than this as the number present in the woodlands where we work is always uncertain (woodland size and character affect the number of squirrels present, as well as the season of study). We have anticipated two separate studies, each comparing three woodlands, each hosting about 50 marked squirrels.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All study designs will be subject to review by the PPLh, a statistician and the AWERB (including an independent statistician), all with a view to ensuring that studies are appropriately but not excessively powered to achieve their aims. Where the evidence required to design appropriately powered studies is unavailable (i.e. variation in dose-response to an agent), expert opinion and experience will be used to arrive at a suitably robust design for small pilot studies, which themselves will inform the design of larger experiments if these are required.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Our use of mathematical modelling permits us to potentially achieve a substantial reduction in the number of animals required to produce a fertility control system in grey squirrels. Modelling allows us to identify, at the earliest moment when the development of contraceptive formulations and self-dosing devices can stop, and when their combination produces a fit for purpose system.

Useful fertility control systems do not have to be perfect, i.e. produce permanent, 100% infertility in 100% of squirrels. Modelling has demonstrated how contraceptives delivered to sufficient squirrels may still a cost-effective method to reduce the number of grey squirrels at the landscape scale. Trade-offs exist in how the properties of the agent, its formulation and its deployment in self-dosing devices can be combined to achieve a useful reduction in the populations of grey squirrels based on fertility control. Modelling will be used throughout the progress of this work to identify when the development and optimisation of agents or formulations (both of which use animals) can be stopped in favour of enhancing

the design of the self-dosing delivery devices or the schedule of deployment.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Much of our work will require the use of live grey squirrels, as it only in this model that the specific reproductive physiology we need to study and modify is expressed.

This is also the target species for our overall aim. However, work on grey squirrels in the laboratory requires them to be brought into captivity, as well as being subject to the limitations of working with a wild animal (distress during routine handling, infrequent and seasonal breeding attempts), and a more tractable model for some of the basic science is available in the laboratory rat. Both are rodents and are likely to share similarities in some physiological processes. As laboratory rats are much more suited to laboratory based research on fertility control, being comfortable in captivity and constantly undergoing reproductive cycles, their use for some of the work is considered a substantial refinement.

Why can't you use animals that are less sentient?

There are no known models for the physiology of grey squirrels that might be considered less sentient. Laboratory rats are no less sentient than squirrels but are at least habituated to husbandry in the laboratory and are more comfortable being handled where this is required for the work.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The work applies refinement and continuous improvement to all elements of the work in this plan.

Specifically, the care and husbandry of the squirrels in captivity is under constant review by a specially convened species specialist team, which includes the NVS, NACWO, squirrel scientists and squirrel technicians who know their animals well. Refinements instituted by this team include minimising un- necessary contact with humans (e.g. remote observation 24/7 using infra-red video recording) and establishing strong routines (times, staff members) to reduce stress to the squirrels.

In addition, captive squirrels have an evolved program of rolling enrichment, for both the facilities within their home pens (branches, hiding places, substrate) as well as feeding (food and presentation of food). This enrichment is considered carefully, as too much change is now thought to be stressful for the animals.

Additionally, most scientific interventions are refined using anaesthesia, which minimises the stress caused to the animal, permits the collection of multiple samples or observations through a single intervention without additional stress to the animals, as well as offering operators the opportunity to achieve protocol steps safely and securely, producing the best care for the animal and best science possible.

The breeding colony of squirrels will be primarily monitored using remote CCTV so that there will be less disturbance and to minimise stress. Only if the squirrels can not be observed via the CCTV or if there is cause for concern will they be psychically checked.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The two collaborating organisations have unparalleled experience at housing and breeding grey squirrels in captivity. This success is produced in part by the unique research facility where the squirrels are housed and provides the space and conditions for their optimum care; and also by constant attention to the refinement of regulated work involving captive grey squirrels and enrichment of their care and husbandry. As part of both of these processes a squirrel specialist group oversees the constant review of the care and use of squirrels to ensure the highest standards are met.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

This plan is delivered as a collaboration between two organisations, both of which are committed to ensuring the most refined regulated work possible. The joint AWERB considers the 3Rs of all studies put forward and has expertise from a range of wildlife scientists, vets and statisticians. Both organisations employ staff who keep abreast of the latest advances in the care and research of laboratory rats. Both organisations also send staff to other organisations caring for squirrels, or studying them, to bring back new ideas to further improve our culture of care for than animals in captivity and improve their experience during the research.

53. Developing new treatments for neovascular eye diseases

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Assessment, Detection, Regulation or modification of physiological conditions in man, animals or plants

Key words

Retina, Blindness, Angiogenesis, Endothelial, Therapy

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To determine whether controlling expression or activity of vascular- acting proteins (either by genetic modification or application of test substances) can prevent or reverse blood vessel growth and neurodegeneration underlying blindness in animal models of Neovascular eye disease, such as wet age related macular degeneration. This will test the therapeutic potential of such substances.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The leading causes of blindness in the UK are diabetic retinopathy and exudative (wet) age related macular degeneration (wAMD). Both diseases are characterised by disruption of the eye vasculature. Despite recent advances in drug treatments using drugs that target

proteins known to influence the eye vasculature (for example anti-Vascular Endothelial Growth Factor (VEGF) and anti-Angiopoietin-2 agents), treatment is still only effective in a proportion of patients, requires regular injection into the eyeball, and at best lasts for a few years before sight threatening disease returns. The burden of eye disease could be substantially reduced by the development of new treatments that are either locally administered as eye drops, or systemically administered as oral agents such as a pill. To bring these treatments to patients requires an understanding of how these blinding diseases are brought about, the identification of new targets for therapies, and the testing of new therapies in models of blindness.

What outputs do you think you will see at the end of this project?

Within the lifetime of this project we would expect to see new treatment approaches developed, and as a consequence development of new inhibitors that could enter pre-clinical testing and production for clinical trial, or, given the progress made on previous licenses, actually moving into clinical trial. The route to this will result in multiple, impactful scientific publications.

Who or what will benefit from these outputs, and how?

In the longer term patients with diabetes and other neovascular eye diseases will benefit. Diabetes affects 8% of the entire population, and diabetic retinopathy is one of the most common complications of diabetes. Patients with wet AMD will also benefit. wAMD affects 10% of people over the age of 70. Together they form the majority of sight threatening diseases. The outcome of this license will be the identification of cell specific druggable targets and development of drugs that stop disease progression and development of blindness, and/or reverse it. We believe this is possible within 5 years from the end of the project license, as work on a previous license (which the applicant worked under) directly led to the development of the current gold standard of care, faricimab, for wAMD and diabetic macula oedema, and development of new therapeutics in clinical trial and in development for clinical trial.

In the shorter term, we expect other researchers and drug development companies and pharmaceutical companies to benefit by being able to more specifically target processes in the eye that lead to blindness.

How will you look to maximise the outputs of this work?

Where appropriate, intellectual property protection will be obtained. We will then proceed to publish the results of the studies conducted under this license through various means: as poster and oral presentations at scientific and patient-focussed meetings, as peer-reviewed publications in ophthalmology and broader medical and scientific literature, and as presentations to pharmaceutical companies interested in developing new therapeutics for blindness. Funding for this research will be sought from government sources, non-profit organizations, and industry partners. This funding will support the development of collaborations, enable participation in meetings, facilitate publication of data, and promote dissemination of our work through collaborative meetings

Species and numbers of animals expected to be used.

- Mice: 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We choose to use adult mice in our research for several reasons outlined in the text.

We employ adult mice to mimic the physiological conditions associated with aging, allowing us to study angiogenesis within a context that mirrors the conditions in older individuals. Mice will be used because of the extensive characterisation of these models of retinopathy. They are the species with the lowest degree of neurophysiological sensitivity in which retinal vasculopathy and neovascularization with similar properties to human disease has been characterised – i.e. in the Choroidal NeoVascularisztion (CNV) model in mice, the new vessels formed are leaky, have similar angiogenic profiles to human disease, and are inhibited by known VEGF mediators. Using mice allows us to genetically manipulate target genes, in temporal and spatial specific manner, that regulate blood vessel formation in the retina in a species that has both many of the same characteristics as humans, but with the lowest degree of neurophysiological sensitivity in which retinal vasculopathy and neovascularization with similar properties to human disease has been characterised

Typically, what will be done to an animal used in your project?

Mice that have been genetically modified so that specific genes can be inactivated (known as knockout mice or inducible knockout mice) will be sourced from other licenses. In some cases to inactivate the gene we will inject a drug (usually either Tamoxifen or Doxycycline) that causes the gene to be switched off.

Mice are anaesthetised for several minutes. To induce a condition that mimics human disease, a laser will be used to make a hole in the membrane at the back of one of the mouse's eye. This involves dilating the pupils with an eye drop and applying several laser spot lesions to the back of the eye, which results in blood vessel leak, growth and inflammation into the eye. We will monitor disease progression by injecting the animals with a fluorescent dye and viewing the back of the eye with a specialized microscopy. This technique is called Fundus Fluorescein angiography, and can be undertaken repeatedly every few days for a number of weeks . This allows us to monitor the size and leakiness of the lesions in anaesthetized mice. We can reverse the anaesthetic agent and mice quickly return to normal behaviour. Following humane euthanasia of the animal, all eyes and relevant organs will be retrieved for ex vivo analysis.

Animals are expected to undergo procedures for up to 6 weeks and will be communally housed with no restrictions or special diets. Mice will be regularly checked for normal health and behaviour.

What are the expected impacts and/or adverse effects for the animals during your project?

In adult mice we have not witnessed weight loss lasting more than 24 hours, but on occasion we have seen formation of a 'milky' lens, which is transient (less than a few hours) and related to the anaesthetic regime we use.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most animals will experience mild severity, as the laser lesion is painless, and the only experience the mice will be aware of is the induction of anaesthesia. There is no pain expected, and, in the animals used, gene silencing is not known to cause any lasting harm or distress. Most animals will undergo multiple imaging procedures, which are separated by days or weeks, and so in total will experience mild severity.

In a very small number of animals (<10%) moderate severity could be expected from multiple injections including intra-ocular injections (tamoxifen, followed by intra-ocular injection of angiogenesis modulator twice a week for 6 weeks and fluorescein injections under anaesthesia).

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Angiogenesis-related ocular pathologies involve complex biological processes, including endothelial cell chemotaxis, migration/matrix degradation, tube formation, and proliferation. These processes also entail the recruitment of marrow-derived angiogenic stem cells and mural cells like pericytes and vascular smooth muscle cells. While we can replicate individual elements of these processes using in vitro techniques, understanding the overall impact of specific factors on angiogenesis necessitates the use of animal models. Moreover, each element in these processes is subject to multiple controls specific to defined tissue environments. Therefore, it's essential to replicate these processes within the tissue affected by the disease under study. Breakdown of the blood retinal barrier is a sign of diabetes that reflects cardiovascular disorder throughout the body. To be able to determine whether the retinal breakdown is happening in concert with, ahead, or behind the systemic changes in vascular permeability we will use non invasive ways to image blood vessel permeability. Such biological processes require interactions with blood flow, physiological levels of oxygen, and the immune system.

Which non-animal alternatives did you consider for use in this project?

As a laboratory we non-animal alternatives to replicate aspects of blood vessel growth in

cell culture models (cell proliferation in vitro cell migration, impedance sensing of monolayers, dye permeability through monolayers, and co-culture assays with endothelial cells and monocytes). These have huge benefit and help us perform preliminary work before commencing animal studies, but no single cell culture assay can recreate all the components of angiogenic remodeling.

Why were they not suitable?

Angiogenesis-related eye diseases are complex biological processes involving a complex interaction between endothelial cell migration, cell division, blood flow and physical forces, and modification of the surrounding tissues. Angiogenic remodeling also involves recruiting cells from the bloodstream. While single elements in the process are reproducible using cell culture techniques, several of which are employed in our own laboratory, it is currently impossible to study the overall impact of any given process on angiogenesis without the use of animal models.

Furthermore, as each of the elements listed is heavily dependent on the specific nature on the site of disease in the body (for example, blood vessel growth in the heart is different to blood vessel growth in the eye) and therefore in order to fully understand the disease we need to study the disease where it occurs - we cannot replicate (yet) the exact cellular environment yet in cell culture studies. However, before undertaking animal experiments, we determine the effect of compounds on cells in culture, in terms of toxicity and efficacy.

We can grow components of interest, for example: Retinal pigmented epithelial cells grown from human eye donors are treated with compounds and then angiogenic factors, cell survival, production of growth factors and cell biological parameters are measured to ensure that the subsequent animal experiments are best informed.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

To enable the minimum number of animals to be used, power analysis and block design of the experiments is used. The least number of animals necessary to definitively show the anti-angiogenic properties (or lack of them) of regulators of angiogenesis will be used, as calculated using power analysis.

For the laser Choroidal NeoVascularisation we are now able to investigate 10-15 agents per year in mice, so plan on using 200 mice per year.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In order to ensure that high quality, reliable and valid data is extracted from the minimum

number of experiments, the ARRIVE 2.0 guidelines will be followed.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Control groups can be used that also reduce the number of animals. This includes using non-invasive imaging and comparing with baseline and ensuring that control groups are appropriate for multiple experiments (i.e. a saline control group in the contralateral eye of 6 experimental animals may be used for the effects of drugs on other animals, as long as there is no likelihood of cross over in the two groups) or use of contralateral eye where appropriate. All experiments are undertaken “blind” with the operator unaware of whether they are administering a test or control substance, and the images are analysed “blind.”

Collection of organs (e.g. eyes, kidneys) under terminal anaesthesia before perfusion with fixative allows preservation of multiple sets of tissues from each experimental animal, thus allowing examination of both histology/ultrastructural determination of pathology and molecular or ex vivo physiological measurements from the same animal. This reduces the total number of animals required as it means that separate animals are not required for fixed as for fresh tissue processing.

For each and every experiment, as part of good laboratory practice, we will write an experimental protocol which includes:

- A statement of the objective(s)
- A description of the experiment, covering such matters as the experimental treatments, the size of the experiment (number of groups, number of animals/group), and the experimental material.
- Statistical expertise is present as the PPL holder has expertise in statistical design and power analysis. Tools used include G Power for statistical power analysis and GraphPad Prism for statistical analysis.
- External statistical advice is also available from the University Clinical Trials Unit in the School of Medicine.

In order to ensure that high quality, reliable and valid data is extracted from the minimum number of experiments, the ARRIVE2.0 guidelines will be followed.

Both sexes of rodent will be used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice will be used because of the extensive characterisation of these models of

retinopathy, and the availability of temporally and spatially specific gene excision or induction. Thus we can excise one gene only from retinal cells, or only from endothelial cells, or myeloid cells, only in adults thus refining the models of gene knockout and ensuring specificity of pathways. The laser CNV model has been refined over the years to be able to investigate processes that cause blinding ndiseases in humans without inducing blindness in mice, or resulting in systemic alterations. Moreover, as we identify further candidate signalling pathways from other research areas we can incorporate additional GM mice into the study, which will allow the most specific and accurate way to study gene function. As the project develops we will incorporate GA approaches to incorporate diabetic and further pathological into our research programme.

Eye drops - Intraocular injection has been developed as a delivery method of large molecules for retinal neovascularisation, as it is the only way of delivering these molecules to the site of action. On a previous license we have now found out how to develop small molecules that we can deliver through the sclera by topical administration hence enabling us to avoid intraocular injection for these types of molecules (but not for antibodies).

Why can’t you use animals that are less sentient?

We use adult mice as there is better disease modelling, and greater translatability than is possible in lower order animals such as zebrafish or frogs. The angiogenic process takes multiple days so animals cannot be terminally anaesthetised for the whole process.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have made a number of refinements to the husbandry and care of the animals to minimise the impact of the procedure e.g, mash diet, nesting material and additional warmth via a cage heat pad as required with increased frequency of cage changing as required.

For the use of novel angiogenesis pathways regulator compounds, pilot studies will be conducted using a stepwise escalating dose with initial dose informed where possible by in vitro studies (protocol 1). Information gained from these pilot studies will inform subsequent administration of substances in the other protocols.

In some animals it may be necessary to perfuse with a fixation agent to preserve biological tissue from autolysis for histological preparation. This will allow us to assess the role of angiogenesis pathway regulators at a molecular level in terms of protein expression such as pro and angiogenic forms of proteins.

Adult free-living animals of assured health and genetic status will be obtained from commercial suppliers or from BSU breeding colonies.

We use injectable anaesthetics as the short time course procedures (routinely under 20 minutes) allows reversal agents to be used, and also eye imaging is impossible on rodents if they are wearing a gaseous anaesthetic mask.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

https://www.nc3rs.org.uk/3rs-resources contains regularly updated information

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will build a culture of care and responsibility within my research group, and contribute to the BSU's community also. I will engage with user groups to discuss and share 'best practice' with all stakeholders across the BSU. I will make use of guidance provided by organisations such as EFPIA and NC3R, alongside UKRI.

Advice on experimental design and statistics is available via the Animal Welfare and Ethical Review Processes. I will also obtain advice from the web-site of www.3Rs-reduction.co.uk.

Home Office

54. Development of Therapeutic Viruses

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Oncolytic virus, Gene therapy, Cancer, Immunotherapy, Vaccine vector

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To develop therapeutic viruses to treat cancer and other human diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cancer affects 1:2 people worldwide. Most people with cancer are treated by surgery, radiotherapy, chemotherapy, or a combination of these methods which have toxic side effects that impact quality of life. Over the last 20 years, drugs that target specific genes or proteins found in cancer cells (targeted therapies) have been developed which have less toxic side effects. One disadvantage of targeted therapies is that not all patients will be eligible for the therapy because their tumour may not have the target molecule the drug is designed to attack. Another disadvantage is that patients who relapse (their cancer comes back) will develop tumours that do not have the target.

More recently, drugs that stimulate the body's immune system to kill cancer cells, known as immunotherapy, have been developed. Immunotherapies include modified immune cells, antibodies, and therapeutic viruses. Therapeutic viruses are trained or engineered to target and infect cancer cells leaving healthy cells alone. How do therapeutic viruses work? When a cancer cell is infected with a therapeutic virus, the virus can multiply in the cancer cell causing it to burst which releases more virus into surrounding cancer cells. Immune cells will clean up dead cancer cells and any left-over virus which are unable to infect normal healthy cells. There are several therapeutic adenoviruses (common viruses that cause mild cold or flu-like symptoms) for the treatment of cancers currently in clinical trials for head and neck cancers, bladder cancer, and colon cancer.

Another way therapeutic adenoviruses can be used to treat cancers and other human diseases is as a gene therapy drug. A gene is a small section of DNA that make proteins which are responsible for different functions in the cell. If the gene is abnormal and not functioning properly, either making too much protein, not enough protein, or none at all, this can lead to disease. Abnormal genes are passed on from parents to children leading to disorders such as haemophilia, cystic fibrosis, and muscular dystrophy. Current medicines can only treat the symptoms and patients will have to take the medication for the rest of their life. Using viruses as gene therapy is a method of introducing a "good" copy of the gene to compensate for the abnormal gene. The changes to the DNA using gene therapy can provide a "once only" treatment to make the disease less severe or cure the disease. The chances of children inheriting the abnormal gene does not change when parents are treated with gene therapy, because the changes to the DNA are not passed on from parent to child. Gene therapy can have a life-changing impact on patients who suffer from inherited diseases.

Adenoviruses can also be used to make vaccines against cancers and other diseases. Cancer vaccines are used for patients who already have the disease which will stimulate the body’s own immune system to attack diseased cells.

What outputs do you think you will see at the end of this project?

The outputs of this project will be:

- data that will be used to file patent applications.
- new knowledge and advancement of the field of adenovirus-based therapies that will be shared in peer-reviewed publications and at national and international conferences.
- data and evidence to advance therapeutic adenoviruses into the clinic to benefit patients.

Who or what will benefit from these outputs, and how?

In the short-term, these outputs will benefit the development of therapeutic cells and viruses and will benefit other researchers in the immunology and virology fields.

In the medium to long term, these outputs will benefit patients, clinical researchers, and doctors as we move the most promising therapies into clinical trials.

How will you look to maximise the outputs of this work?

To maximise the outputs of this work, where possible we will publish in open-access

journals and share data including unsuccessful approaches, share and use tissue samples across various projects, and share best practice with collaborators. We will also present work at national and international conferences.

Species and numbers of animals expected to be used

- Mice: 3000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use laboratory mice, both wild type and genetically modified, as they are the most reliable mammal for mimicking the human immune system, which is vital to our work. Genetically the mouse is very similar to the human in terms of their genetic makeup and are easily genetically manipulated to replicate the human. As they mimic the human immune responses, they are also a reliable model of a cytokine storm enabling us to determine the safety of the drugs and doses we are administering.

We will use both adult male and female mice, unless the cancer we are studying is sex specific (e.g., ovarian cancer, prostate cancer). We choose adult mice over juveniles as we require a fully functional immune system for our studies.

Typically, what will be done to an animal used in your project?

We will be using genetically modified mice which are immunocompromised from commercial sources, which will be held in groups to ensure good socialisation and sterile conditions.

Some mice will have a tumour induced by having tumour cells injected under the skin (subcutaneous) of the flank of the animal. These tumours will be allowed to grow and will be measured up to 3 times per week. The tumours may then have a virus injected into them to assess the efficacy of the treatment, at a dose and schedule we would expect to deliver in the human patient. Once the tumour reaches the maximum size required to answer our scientific questions, which is no larger than 1200mm3 the mice will be humanely killed.

Cancer cells can sometimes move from the main tumour site to another part in the body known as a metastasis. Cancer cells will often travel through the blood stream to reach organs farther away like the brain or move to organs that are close to the original tumour. To study the mechanics of metastasis and understand how to treat metastatic tumours, some mice will have their tumours induced by injection of cancer cells into the blood stream or into the abdomen.

Some of our tumour cells may be engineered with a fluorescent or luminescent protein that will be visible under a purposely designed fluorescent imaging camera.

The glow is enhanced when the tumour is of a larger dimension. This imaging is performed under general anaesthesia.

Some mice will be exposed to a low dose of irradiation to suppress their immune response.

This is to prevent the immune system attacking the implanted human cells and to allow them to grow into a tumour. We typically give the appropriate dose over several small doses, known as a fractionated dose, which minimises the adverse effects the animal may experience.

Where hormones are required for a specific cancer to grow this can be administered in the animals drinking water.

Some mice will have viruses administered by either an intravenous or intraperitoneal injection which is not expected to cause the animal any distress. We may also inject viruses directly into the tumour. All the treatment doses and schedules will be similar to how would expect to deliver treatment to the human patient.

Some mice will have small blood samples taken over a short period time so we can evaluate how effective the treatments are. This may be continued for up to 10 weeks after the treatment phase is completed.

At the end of the experiment, all animals will be humanely killed.

What are the expected impacts and/or adverse effects for the animals during your project?

Tumours injected into the flank of the mouse may break the surface of the skin which may be treated with a barrier cream. Treatment will be discontinued after 3 days, and the animal humanely killed if there is not improvement. Oher tumours may develop a larger and deeper ulceration of the tumours and these mice would be humanely killed upon detection.

Tumour cells injected intravenously or intraperitoneally may migrate to other organs, such as the lungs and result in the animal displaying laboured breathing.

Some of the viruses and treatments we inject may cause transient illness such as weight loss of up to 15%, piloerection, hunched posture, and lethargy. Typically, this is an involuntary reaction to shock from the virus itself and only occurs at higher doses. We expect these signs to resolve within one hour of the virus being given.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

45% mild 55% moderate

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In the laboratory, we use human diseased and normal cells to study how well our therapeutic viruses specifically kill diseased cells. But cells in a petri dish do not replicate the immune environment the therapeutic virus will encounter once it is injected into the body. Using mice to model disease will provide evidence that the killing we see in the controlled environment of the laboratory is translatable to a whole living system.

While we can study how immune cells react to the virus in the laboratory, we cannot mimic how the complete immune system will react to the virus. The similarity between the mouse and human immune system will let us predict the safety and type of immune response that may result from the therapeutic virus. We can also use this information to determine possible safe dosing in humans.

Which non-animal alternatives did you consider for use in this project?

We use immune cell and tissue cultures to assess the most effective viral candidates to move forward into the mouse.

Why were they not suitable?

Immune cell and tissue cultures are not sufficient to evaluate the effects withing a complex immune environment and tumour microenvironment. In addition, the biodistribution of the virus throughout the body is more appropriately evaluated in mouse models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We will use data from previous experiments to determine the appropriate number of mice to use in animal studies. If we have not done a similar study before, we conduct pilot studies using small numbers of mice (i.e. 3-4 mice per group) to test the best study design before designing studies with larger numbers of animals.

We are aiming to develop 10 therapeutic viruses over the course of the project, with each virus requiring up to 5 studies. Our estimated group size for these studies is 36.

For developing and characterising models of disease we aim to evaluate four models over the course of the project. Our estimated group size for these studies is 20.

To test the efficacy of our therapeutic viruses we anticipate up to 20 studies over the course of the project. Our estimated group size for these studies is 50.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have a multi-disciplinary team (biologists, immunologists, virologists, and biostatistician) that discuss study ideas and design to develop an appropriate study for our research question. We use the Experimental Design Assistant (EDA) from the NC3Rs to design the studies which addresses all the different variables, controls, and biases. Then an extensive study plan is written and circulated to different scientists for further advice and comments before approval by a Study Director

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For therapeutic viruses not yet tested in living systems, we do pilot studies to confirm safety, tolerability, and uncover any unexpected adverse effects. We also conduct pilot studies when characterising new models of disease to determine if it is appropriate, determine the time to humane endpoint, and to uncover any unexpected adverse effects.

From these pilot studies, we establish the range of tumour measurements and levels of proteins measured in the blood which is used for power calculations to determine appropriate sample sizes for larger studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use genetically modified mice into which we inject human tumour cells subcutaneously under the skin at the flank. The tumours can easily be seen and measured. Mice will briefly be anaesthetised for the subcutaneous injection to ensure that tumour cells are implanted in the correct area, minimises any pain and distress for the mouse. Mice will experience only temporary pain, suffering, distress, and no lasting harm from handling during tumour measurements.

For blood cancers and metastasis, we will inject tumour cells in the tail vein or in the abdomen. Tumour growth will be measured by imaging with a special camera as the animal is sleeping under anaesthesia or by taking weekly micro blood samples.

These methods only cause temporary pain, suffering, and distress from handling during injections, blood sampling, and imaging under anaesthesia. Monitoring by imaging or blood sampling means that we can get data without having to let the animal suffer by letting the disease progress.

The therapeutic adenoviruses to be used in animal studies have been carefully selected for its ability to target human cancer cells. Though the virus primarily infects human cancer cells only, the presence of virus particles can still lead to an overreaction by the immune system, such as it occurs in humans. To refine our dosing strategy, we first give a low dose of virus to train the immune system to get used to the virus, before giving the higher doses necessary to kill cancer cells.

Each virus will not have the same effect on the immune system; therefore, we need to test and refine the dosing strategy for each new therapeutic virus.

Why can't you use animals that are less sentient?

Mice are the least sentient animals that can be used because they have a similar immune system and have comparable immune responses to humans. Mouse models will allow us to study the effect of therapeutic viruses on the immune system and on the cancer cells.

During the study, it is important that animals are active and awake because anaesthesia can inhibit the production of critical immune proteins that we want to measure. If we did these studies under terminal anaesthesia, we would not be able to test our virus on actively growing tumours and see if the virus is making a difference.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have extensive experience in developing therapeutic viruses using mouse models. During treatment with the virus, there is the largest potential for adverse events especially after injection which can cause a shock to the immune system.

Mice are monitored for 30-60 min immediately after injection for any sudden effects of shock. If mice show signs such as hunched posture, abnormal activity combined with heavy breathing, or fur standing up, that do not resolve within 60 min after injection, we will humanely kill the mouse. We know that mice will lose weight during the first week of treatment but typically then begin to increase weight again especially in the days after treatment.

Therefore, mice are monitored daily for weight and if they lose more than 10% of their starting weight and continue to lose more over the next 48h, mice will be removed from the study and humanely killed. Mice will be given gel diet/water placed on the floor of the cage for extra nutrition within easy reach.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use:
Guidelines for the welfare and use of animals in cancer research for best practice in tumour induction and growth.

LASA guidelines for best practice for routes and volumes for the administration of substances. ARRIVE and PPREPARE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are constantly reviewing the literature for best practice and new techniques including non-animal alternatives to do our studies. From other scientists at national and international conferences, seminars, and meetings, we learn techniques and methods which we adapt for our purpose. We will consult our local NC3Rs representative and animal care staff to learn refined methods and share any refinements we employ.

55. Fish Physiology in the Anthropocene

Project duration

5 years 0 months

Project purpose

- Basic research
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Temperature, Hypoxia (low oxygen), Pollution, Metabolism, Cardiac function

Animal types	Life stages	
stickleback (Gasterosteus aculeatus)	embryo, neonate, juvenile, adult, aged Zebra fish (Danio rerio)	embryo, neonate, juvenile, adult, aged
Brown Trout (Salmo Trutta)	juvenile, adult, aged Rainbow Trout (Oncorhynchus mykiss)	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The proposed research will investigate how environmental change in isolation and in combination affect key life history traits like swimming, metabolism and behaviour in fish. As cardio-metabolism underlies these key performance traits, we will also study how the environment (temperature, hypoxia, man-made pollution) impacts the fish heart.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Climate change represents a significant challenge for resource managers (e.g. the

Environment Agency) who require advice on the resistance, resilience and adaptability of fish populations to environmental change. Fish are ectotherms (i.e. cold-blooded) and can adapt to a range of environmental conditions including acute (minutes to days) and chronic (weeks to months) changes in temperature, dissolved gases (oxygen and CO2), and even in anthropogenic (human-made) pollution.

However, there is concern that because such changes are occurring more rapidly and in combination, that while these populations may survive, they may not thrive.

This is called lack of fitness. This lack of fitness means they may not have the ability to perform activities required for lifetime success like migration, courtship, or withstanding a further environmental challenge. The purpose of this application is to study how fish fitness is impacted living in the challenging environment of today's rivers. Through this work we hope to implement better and more timely resource management for wild and for managed populations.

What outputs do you think you will see at the end of this project?

We expect this project to produce new data that will form the scientific foundation for both academic publication, and public and stakeholder engagement. Advancing knowledge on multiple environmental stressors across life stages in riverine fish (as opposed to model species) will be of interest to river and lake anglers, to canal and rivers trusts, and national parks managers. It could also be used to hold current water management companies (e.g. water companies) accountable. These are realistic benefits as our current work on microplastics in urban rivers has led to water companies having to disclose untreated sewage release events as part of a public inquiry.

Who or what will benefit from these outputs, and how?

Fishes are the primary beneficiary. We have chosen the species for this study in collaboration with local angling and rivers trust groups to represent fish that live in different parts of the river (e.g. benthic (bottom feeding) versus open flow). We hope that our findings contribute to river species welfare through initiatives run through these partners like the building of tree-shade or cool refuges. Healthier water ways improve the well-being of greenspaces for humans, animals, and ecosystems. The **short** term benefit will be in defining integrated limits of environmental tolerances for a number of fish species across life stages. In the **medium** term this data can used to create more bespoke management models. Indeed, policy makers, environmental lobby groups, water and environment regulators will benefit from this project as they will be able to use the findings to make decisions for the benefit of the fishes. The **longer** term benefit of the knowledge generated from this license will improve survival and welfare of fish populations both in wild systems and in aquaculture.

How will you look to maximise the outputs of this work?

We will continue to work with local angling groups, friends of the canals/waterways, and Rivers Trusts to improve the condition of the water fish live in. We will publish in open-access journals, presentation at scientific meetings and make our resources available to other researchers including data and tissues. Our findings may include negative results and will publish and disseminate these with the same commitment and strategy we use for positive findings. Our work could impact policy and will be active in this arena and also

work together with local policy providers to ensure findings research the public realm. We routinely engage the public through outreach and widening participation activities and will ensure findings from this project are shared here to inspire the next generation of enviormental bioloigsts and fish scientists.

Species and numbers of animals expected to be used.

- Brown Trout (Salmo Trutta): 50 (or rainbow trout)* will use either Rainbow or Brown depending on availability, not both
- Rainbow Trout (Oncorhynchus mykiss): 50 (or Brown trout)* will use either Rainbow or Brown depending on availability, not both
- Other fish: No answer provided • Zebra fish (Danio rerio): 2500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Choice of species:
Temperate Riverine fish: Trout (rainbow *Oncorhynchus mykiss* and/or brown *Salmo salar*) will be used as they are a fish that feeds on the surface of the river, are native to UK waters, and are sensitive to environmental stress. They are easily attainable from fish farms and we have more than 25 yrs of experience working with this fish. Common **minnow** (*Phoxinus phoxinus*), **stoneloach** (*Barbatula barbatula*), three-spinned **stickleback** (*Gasterosteus aculeatus*) are all common fish in UK waterways. We have measured their abundance in urban rivers and the presence of microplastics in their bodies and environmental pollutants in their habitats. These 3 fish feed differently in the water column and thus may have variable exposure to pollutants. For example stone loach feed in the sediments whereas the stickleback tend to feed from the surface (as adults). All 3 species are small in size and are readily held in tanks, and are easily attainable from pond shops.

Carp-like fish: Zebrafish (*Danio rerio*) are a model organism whose genetics can be easily manipulated. We will use them in this project to probe questions where altered physiology through CRISPR technology allows deeper understanding of environmental tolerance landscapes. The work with the zebrafish builds directly on the findings from the my previous PPL. Carp species like goldfish (*Carassius auratus*) or common carp (*Cyprinus carpio*) are exceptionally tolerant of environmental stressors - sitting at the other end of the tolerance spectrum from trout. These are easily attainable from pond shops.

Choice of life stage:
This project will work across life stages from **embryo/larvae**, to **juvenile**, to **adult** and **aging fish**. It is clear from the literature that embryonic and larval stages are more vulnerable to environmental stressors than adults and thus we must conduct studies at these ages to understand the vulnerability of fish populations. Moreover, as the developmental environment impacts later life fitness though a process known as *developmental programming*, it is vital to follow fish that have been exposed to stress in early life, into adulthood. We have separate protocols for developmental programming

work and for life-stage specific exposure work.

Typically, what will be done to an animal used in your project?

There are 3 parts of this application that detail what will be done to a fish in this project. Each are described below.

The rearing conditions for embryonic fish (developmental programming exposures and holding/maintenance conditions for juveniles/adults/aging adults (environmental exposures).

The Environmental and Behavioural Tests that a fish will go through to understand environmental tolerances. For example, assessing min/max metabolic rate; swimming (fish only, not embryos); thermal tolerance test; hypoxia tolerance test; and behavioural tests.

The cardiovascular monitoring, blood taking or substances administration at various time points across the life stage to determine physiological mechanism underlying environmental tolerances.

Rearing Water and Holding Water Conditions:
The aquatic habitat is an integrated environment, comprised of multiple changing environmental parameters. Consequently, fish simultaneously experience changes in several environmental factors including water temperature, dissolved oxygen levels and anthropogenic (man-made) pollutants. These environmental conditions can act singly and synergistically on the physiology of fishes. We will rear (during embryonic development) or hold/maintain (in protected life stages) fishes under varying temperature and oxygen levels, with or without pollutants and microplastics.

The Challenge Tests:
To understand how a rearing/holding environment impacts the ability of a fish to tolerate an acute environmental challenge, fish will experience a set of 5 challenge tests none of which are expected to cause lasting adverse effects.

Maximum and minimum metabolic rate: where oxygen consumption is used as an index for metabolism.

Swimming tests: where fish swim voluntarily in a swim-flume (fish treadmill).

Thermal tolerance tests: where fish will experience an increase in water temperature until they loss equilibrium, from which they recover well.

Hypoxia tolerance tests: where fish will experience a decrease in oxygen levels until they loss equilibrium, from which they recover well.

Behavioural Tests: where fish will have their behavioural monitored whilst making decisions, exploring new arenas, or choosing a thermal preference zone.

Tagging, Cardiovascular monitoring, administration or withdrawal of substances
Tagging: Fish may be tagged for individual identification which is not expected to have any lasting adverse effects on the fish.

Monitoring/Instrumentation: Fish will be anaesthetized by immersion in water containing anesthetic and then placed on a surgery table with gills irrigated with a maintenance dose of anesthetic and covered to keep the body damp. Here cardiac parameters can optionally be measured non-invasively using an ultrasound, or more directly with ECG monitoring electrodes inserted just beneath the skin on either side of the heart, or with cannula and flow probes in larger fish.

Administration of substances: In some instances, the effect of the environment on growth and metabolism will be investigated following an intramuscular or intraperitoneal injection of growth regulators or growth assessors in adult fish. These could be substances that increase metabolism like adrenaline, or substances that help us record fuel use during growth like insulin. Such substances can be administered to embryonic fish via the water or directly via injection into the yolk.

Withdrawal of blood: Blood may be taken to assess for hormones, metabolites etc. Usually there will be a single blood sample taken not more often than once a week under anesthesia.

All fish will be humanely euthanized at the end of project.

What are the expected impacts and/or adverse effects for the animals during your project?

Our environmental exposure groups have been chosen based on our monitoring work in the rivers and water ways around the Establishment. Our aim is to choose experimental group that reflect the conditions fish are currently experiencing in the wild.
We do not expect long term welfare issues with our holding or rearing conditions. In instances where fish are exposed to environmental pollutants alone or in combination with changes in other environmental parameters, we may expect slower growth and reduced activity. However, oxygen, temperature and pollution change routinely in their natural environment across the seasons and with run off into the rivers changing growth and activity as a normal part of the seasonal cycle. We do not expect the rearing conditions will have adverse effects beyond growth and activity levels. But to control for any potential adverse effect, we will screen our combination of environmental conditions during the pre-protected life stages, which are also the most sensitive life stages in fish. We will monitor how the embryos develop and any conditions that cause abnormal development will not be used with protected stage fish.

None of the challenge tests are expected to have lasting impacts on welfare. We also note that some of the 'tests' like the behavioural investigation of a novel object are used as enrichment. However, we recognize that undergoing multiple environmental challenge tests over the course of a lifetime will have cumulative effects and to minimise any adverse effects accruing we will allow ample time (~3 months in most cases) between sets of environmental challenge tests.

All experiments where cardiac function is recorded will be conducted under non-recover anesthesia to avoid any adverse effects.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The rearing and holding conditions and the behavioural trials are expected to be sub-threshold and 80% of the fish will experience these conditions.

Of the 100% of all fish (80% of which will not be in 'standard conditions') 60% will under go challenge tests that are mild. 40% will undergo the 2 challenge tests that are moderate.

What will happen to animals used in this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This work has to be carried out on fish because, as aquatic ectotherms, their vulnerability in relation to climate change differs from other animal models (i.e. terrestrial vertebrates or invertebrates).

Additionally, fish are the prime beneficiaries of the output from this work, understanding tolerance in relation to fish biology is paramount Although part of the research in this program of work employs non-invasive techniques, such measuring metabolism by recoding oxygen consumption, and tissue levels studies (molecular, biochemical, genetic) following humane killing, to assess mechanisms limiting thermal tolerances, it is necessary to measure the integrated environmental thresholds of the whole animal.

It is important to remember that thermal tolerance varies with level of biological organization: Complex processes requiring peak swimming performance, like migration and reproduction, will have lower thermal tolerance thresholds than single cells or isolated organs. Thus, work must be conducted over a range of levels so that mechanism can be understood at the molecular level and functional consequences for lifetime fitness can be appreciated at the whole animal level.

Indeed, advising policy makers on thermal tolerance thresholds arrived at from isolated tissue studies alone may well underestimate the sensitivity of the system.

The genetically altered zebrafish provide the most feasible way to address the underlying mechanism for growth and development limitations under various environments.

Which non-animal alternatives did you consider for use in this project?

We have previously provided data for modelling studies of cardiac function in response changes in temperature and to polyaromatic hydrocarbons. Any relevant data from the

current project will be used to parameterize these models.

Unfortunately, whole animal simulation models do not exist.

Why were they not suitable?

They do not exist - modeling whole organismal responses is currently not possible.

But should some be developed we would adopt to reduce animal use.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

A statistician helped us with calculations using typical variations from our previous data. We will use different species to ask specific questions relevant to the environment. For example, if we are interested in benthic (from the riverbed) uptake of microplastic from the sediments into a fish we would use stoneloach as they feed in the benthos and this exposure route is more relevant to them than a trout. As we are interested in co-occurrence of different environmental stressors, we will have treatment multiple groups and as we are interested in interactions, which can be harder to detect, we estimate the need for larger sample sizes. For example if we wanted to look at the interactive effects of microplastic exposure with temperature and a pollutant, we will need 6 treatment groups. Sample sizes for our experiments are estimated from past experiments. Calculations typically show that we need group sizes of 10 to achieve the quality of results we need. We've used our annual return of procedures data to estimate the number of animals that we will need to use for breeding.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We discussed our design with a statistician such that we used the least number of animals whilst adequately powering our studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will evaluate the effect sizes and samples sizes for each species as we go and revise where applicable. For some species where less is known about their responses to environmental change we have based sample sizes on responses know from more common species. We will check these assumptions routinely.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Our project is trying to understand the implication of climate change on fish and all of the environmental conditions employed in this study are already faced by fish in their natural environment. There are >40 fish species and this project has chosen 6 different species who either occupy different but relevant environmental niches (e.g. benthic stoneloach vs surface feeding stickleback) or have different tolerances to their environment (e.g. hypoxia tolerant carp v hypoxia sensitive salmonids).

The project also involves zebrafish as a genetically amenable model fish. This allows us to specifically define certain genes or pathways that are involved in fish environmental tolerance. We may instrument some fish to allow us to understand the role of the cardiovascular system in environmental tolerances and this will always be done under appropriate anesthetic.

Why can't you use animals that are less sentient?

Our project is for the benefit of fish and designed to help us understand how they respond to the multiple and simultaneous stressors living in the an era of climate and environmental change entails. Cell culture or invertebrate models would not provide the fish-specific, or integrated whole animal performance information that this project aims to gain.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will monitor fish during all protocol steps and if we recognize an occasion where a lower threshold can replace current ones, we will discuss with our animal care facility and implement where appropriate. An example is using agitated swimming responses rather than loss of equilibrium responses to an acute thermal challenge. Ageing animals will be carefully monitored by staff trained to work with aged fish.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I keep on top of resources and guidance from the NC3Rs and the Laboratory Animal Science Association and will keep abreast of symposia relating to wild fish welfare.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

As project license holders and for all personal working under a Home Office license, we will attend lectures and workshops put on by the NC3Rs, our local NC3Rs manager, and our animal care unit.

56. Generation and characterization of neurodegenerative disease relevant models

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Genetic Diseases, Synthetic Rescue, Rodent Models, Model characterization, Neurodegeneration

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to identify genes and genetic networks that are involved in human genetic diseases (for example neurodegenerative diseases).

Specific purposes of this project are:
Import and/or generation as well as maintenance of disease models (mouse models which carry the disease of interest)

Characterization of these imported and/or newly generated disease models (mouse models which carry the disease of interest) to validate genetic targets and/or determine a disease relevant phenotype (observable physical properties that are indicative of and relevant to the disease)

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This work will generate and identify new disease relevant models and phenotypes which will then be utilized as part of another licence to develop novel therapies for treatment of genetic diseases.

Without a full understanding of the disease phenotype and the relevance of certain genes in the context of this disease phenotype, it is not possible to develop new therapies for treatment of genetic diseases, many of which are affecting children or young adults with cataclysmic effects on both patients and their families.

Many of these diseases currently have limited to no therapeutic options with existing therapies showing limitations such as incomplete response, relapse or emergence of resistance.

Additionally, it will help bridge the current knowledge gap for many of these diseases as well as enable impactful translational research.

What outputs do you think you will see at the end of this project?

The outcome from this project will be the identification and validation of novel therapeutic targets which will enable us to develop novel therapeutic drug candidates (under a different PPL).

New disease models will be created and readily available mouse strains will be investigated further, providing novel disease relevant phenotypes. We will gain new insights into disease mechanisms and better understand the relevance of certain genes and genetic networks in this disease.

Data obtained from our work will be published and/or presented at research meetings, thus advancing knowledge of disease processes and drug discovery technologies in the scientific and clinical community.

Who or what will benefit from these outputs, and how?

This project aims to identify novel, disease relevant targets and phenotypes which can then be utilized to develop new therapies (under a different licence) which will benefit patients that currently have limited or no treatment options available.

Data on the most promising models and disease relevant targets will be communicated to the wider scientific and clinical community typically first at conferences and then as peer-reviewed journal articles.

Generated disease models (including genetic crosses) will, if possible, be made available to other researchers in order to prevent duplication of breeding efforts.

How will you look to maximise the outputs of this work?

In order to maximise our outputs, we will collaborate with experts, both academic and industrial, in these disease areas and work closely with patient advocacy groups to determine the best way forward to successfully develop novel disease relevant models.

For the technical work, we will work closely with the Establishment's Laboratory Animal Resource Management, including Named Veterinary Surgeon (NVS), Named Animal Care and Welfare Officer (NACWO) and technicians and discuss experimental approaches from both scientific and technical aspects to maximise the output and probability of success for our program.

We plan to disseminate new knowledge by publishing it in open-access journals, presenting at scientific meetings and making resources available through collaboration with experts in these disease areas. Investigations that led to de-validation of potential drug targets will be considered for publication.

Species and numbers of animals expected to be used

- Mice: 17800

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice will be used because they are considered to be the species with the lowest sentience that have well-characterised disease models available for target identification, validation and characterization. They share sufficient anatomy and physiology with humans to be used for the identification of novel therapeutic targets.

Mouse models have been extensively used in target identification/validation and detailed standard protocols are available. For example, detailed phenotyping protocols are available, allowing identification of disease relevant phenotypes.

Young mice may be used for disorders that present early on in life (pediatric diseases).

Animals may be humanely killed at different ages to provide primary tissues and organs for ex vivo assays, for example for omics analysis (such as proteomic and/or transcriptomic analysis) or where no suitable immortalised cell lines are available or the use of human induced pluripotent stem cells (iPSC, a type of cell that is able to develop into many different types of cells or tissues in the body) is inadequate.

Typically, what will be done to an animal used in your project?

Existing mouse strains will be imported where possible. Some new mouse strains will be created and different strains of mice will be bred together to either generate new disease relevant models or determine if a genetic modification in one strain stops the signs of

disease in another. We will study these strains to determine their phenotype as well as to see if they produce offspring at the usual rate and whether these develop normally.

Individual mice from these strains may be observed over several weeks and potentially months of their lives and have their weight and size measured.

Procedures may be carried out on these mice at different times during their lives, such as blood sampling, phenotyping (behaviour or image based tests), imaging or substance administration (such as but not limited to transgene inducing or deleting agents or imaging reporters). Post mortem tissue harvest (for post mortem analysis) will be performed to determine differences between disease strains and their wild type littermates.

Animals will experience mild and transient discomfort from blood sampling.

Disease relevant phenotypes will be identified by imaging (for example bioluminescent, fluorescent or MRI imaging) or behaviour tests over several weeks or months of their lives. Animals used for imaging are not expected to experience any more than mild and transient discomfort from these procedures.

Non-invasive behaviour tests will be carried out to determine potential disease relevant phenotypes. We do not expect them to experience any more than mild and transient discomfort from these procedures.

Typically, animals will experience mild, transient pain and no lasting harm from administration of substances by injection using standard routes (for example: into a vein, under the skin or into the abdomen).

Final procedures, such as harvest of tissues, will be undertaken under non-recovery anaesthesia where the animals will only be aware of the anaesthetic being administered and may experience mild distress and no pain.

If an animal becomes sick unexpectedly it will be humanely killed and examined to see why it was displaying these symptoms. If possible, samples will be collected for further analysis.

All animals are intended to be group-housed. Single housing will be minimized by regrouping animals from within the same treatment group where possible. In cases where aggression means social housing is impossible on welfare grounds, the mice will be housed on their own with increased environmental enrichment.

What are the expected impacts and/or adverse effects for the animals during your project?

Many mice will suffer no adverse effect during the project.

Most mice will experience no more than transient feeling of pain or suffering.

Some mice will undergo surgical procedures for example for embryo transfers. They are expected to recover quickly and will be given painkillers and post-operative care just like people recovering in hospital.

Procedures for substance administration (for example for imaging) are well established and will cause transient pain and no lasting discomfort.

Animals may develop at a slower rate and/or have developmental abnormalities (for example curvature of the spine) or other phenotypes (for example loss of coordination or seizures) indicative of the disease they model. These effects may develop gradually over weeks and will not be allowed to prevent the mouse from feeding and drinking.

Some animals may show signs of ageing earlier. There may be gradual weight loss, over several weeks, and loss of fat and muscle mass.

Some mice will form tumours as part of their disease progression. Tumour growth will be monitored and will not be allowed to stop mice carrying on with normal behaviour. For short periods, the body weight of the mouse may increase or decrease in the presence of a tumour. Mice may move differently or have uncoordinated movement. Breathing may be more difficult. Skin could become more irritated in the presence of a tumour.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

About 30% of the mice will be subthreshold. About 50% of the mice will be classified as ‘mild’.

About 20% will be classified as ‘moderate’ severity.

Approximately 2% of the mice will experience procedures under non-recovery anaesthesia.

What will happen to animals used in this project?

- Killed
- Kept alive at a licensed establishment for non-regulated purposes or possible reuse
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Mice will be used because they are the lowest vertebrates that have well characterised models for target identification and validation.

The disease areas for which we are identifying new disease relevant phenotypes and targets rely on complex tissue crosstalk with many different cell types interacting and communicating with each other. Many of these processes are complex and currently there is no in vitro system that can reliably replicate the in vivo environment.

However, we will perform in vitro experiments in relevant cell models, such as experiments that determine how different genes work together to make a disease better or worse, before progressing to animal models.

Which non-animal alternatives did you consider for use in this project?

Extensive analysis without using mice is integral to the project and is always the first option considered when new biological areas of interest are identified.

We consider and in many cases are utilizing the following to minimize the number of animals required for each study:

- human and/or epidemiological data analysed via our Human Genetics Team and used to guide and inform the different projects.
- in vitro methods such as cell culture or three-dimensional in vitro systems (such as organs on a chip [systems containing cells grown inside microfluidic chips designed to better mimic human physiology] and organoids [three-dimensional miniaturized versions of organs or tissues that are derived from cells with stem potential and can self-organize and differentiate into 3D cell masses, recapitulating the morphology and functions of their in vivo counterparts]) will be used in conjunction with all ongoing projects. For example, we are actively supporting the development and use of robust new organoid models by collaborating with pioneers in the field for generating cerebellar models from human iPSCs.

Why were they not suitable?

We will utilize all the above-mentioned alternatives throughout the duration of the licence wherever possible to complement the in vivo work.

However, due to the complexity of the disease areas we still rely on using in vivo models to enable target validation as the cellular and tissue context can influence effects of target modulation.

- Limitations to the in vitro methods are as follows:
- Even though great advances have been made using in vitro methods, they are still not capable of fully recapitulating the complex tissue crosstalk that is present in vivo. For example, for complex disease areas (such as neurodegenerative disease) development of iPSCs into relevant cell types is not always possible or if successful result in a more immature cell type that does not fully mimic the cell types and complex microenvironments found in vivo. Additionally, primary cells are difficult to obtain. Especially due to the time it takes to obtain these cells from a deceased patient until they are established in the laboratory, their cell signalling and epigenetic markers are often altered which makes them not comparable to cells studied within the in vivo microenvironment.
- Several different types of three dimensional in vitro systems (for example organoids and organ on a chip systems) have been considered but to date only limited systems exist. In addition, they do not yet fully recapitulate the in vivo microenvironment.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We will use appropriately designed and analysed animal experiments that are robust and reproducible.

Calculations are based on discussions with the Laboratory Animal Resource support team, internal project teams and statisticians as well as the previous experience of the licence holder. Statisticians are considering variations reported in the literature to calculate minimum numbers of animals to be used whilst ensuring that experimental objectives can be reached and the results are meaningful.

Group sizes for each specific study will be confirmed to be appropriate by the Statistician before initiating the work.

We anticipate running between around 5 programs a year, each of which will require 2-3 disease relevant mouse models throughout the study. Based on the disease areas we focus on, about two thirds of models will be rederived and about a third will be new models (generated either via crosses or by gene editing).

The breeding protocols will be used to generate experimental cohorts for Protocol 6 on this licence but also for experimental protocols in another PPL. We also took into consideration the fact that some strains, for example ATM-knock out strains, are homozygous infertile and hence we will require larger breeding cohorts to generate the homozygous animals needed for phenotypic and experimental analysis.

Moreover, from experience working with some of these strains at CROs, we know that we require between 5 to 10 animals of each sex and genotype for phenotypic analysis.

Controls and dose levels will be chosen based on in vitro data generated in our laboratories or obtained from published literature. If these cannot be reliably determined, we will perform small pilot studies first.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In vivo experiments are not carried out until there is sufficient evidence from testing in the lab as well as in many cases utilizing our Human Genetics platform. Animal sample size calculations are made to ensure as few mice as possible are used whilst ensuring experimental objectives can be reached. We employ the help of statisticians and expertise of the establishment’s support team to plan our experimental design, practical steps and statistical analysis, taking into consideration randomisation, blinding, sample size calculations and appropriate statistical analysis methods. Additionally, online resources from trusted and experienced organisations provide software and templates will be utilized.

Model generation will be replaced by import of exiting models wherever possible.

When creating new lines, we will use optimal methods for design and production. For example, it is our intention to create new GA mice using CRISPR technology wherever possible, which significantly reduces the number of animals required both for surgery procedures and later during breeding.

The accumulated in vivo experience from both the PPL holder, the in vivo team as well as the Laboratory Animal Resource Unit will be used to continuously improve in vivo technologies to reduce the number of animals required for each study.

For investigation of new potential drug targets, we review the literature for the relevant potential drug target to understand their prevalence and possible range of target modulation in vivo and use this information to modify our standard study designs. We will conduct in vitro experiments followed by in vivo pilot studies to verify the suitability of the model and to further optimise the study design, such as group size, study period and sampling timepoints.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Pilot studies using small number of animals will be performed wherever suitable and possible.

Where possible we shall import existing mouse lines from publicly available repositories rather than generating new ones. In general, we will order lines in as frozen sperm or embryos for recovery. This will reduce the number of mice used for breeding between facilities as well as remove the harms related to the stress mice are under when in transit.

Wild type animals will be purchased from reputable commercial sources. Breeding these in sufficient numbers as part of this licence would result in a large excess which we wish to avoid.

All disease model lines, both imported as well as newly developed, will be archived so that they may be distributed to other researchers worldwide. This will reduce the number of animals used globally, as fewer animals will be required to re-generate these archived lines.

Efficient breeding will take place due to dedicated staff and use of the electronic database available from the Laboratory Animal Resource Unit.

Computer modelling as well as in vitro studies will support all in vivo work.

At the end of the experiment, we will harvest as many tissues as possible at post-mortem. If we don’t need to analyse the tissues immediately, we will freeze them and potentially even make them available to other researchers working on similar questions.

Wherever possible, we endeavour to publish data generated from our research in scientific journals available to the whole scientific community, reducing duplication of production resources and phenotyping procedures elsewhere.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mouse models have been extensively used for the identification and validation of new therapeutic targets and disease relevant phenotypes and detailed standard protocols are available in the literature.

Some neurodegenerative disease models may require a long study period with highly variable results, thus requiring higher numbers of animals which suffer gradual and long-term disease-related symptoms. This will be considered in the study design and the animals will be monitored closely to avoid any discomfort and animals will be culled if they show signs of suffering.

Various different phenotyping approaches (behaviour and/or imaging) will be used to assess disease phenotypes or bioavailability of compounds. These studies will be designed with consultation from imaging experts to avoid unnecessary stress and discomfort for the animal.

Why can't you use animals that are less sentient?

To demonstrate the importance of the identified targets on the relevant disease phenotype, we need to rely on in vivo models due to the current limitations of existing in vitro systems in recapitulating the naive cell environment.

Mice and humans have the same basic organ systems, skeleton and reproductive cycles. These similarities, coupled with the technology available to manipulate the mouse genome, make the mouse the best model to mimic human diseases. Using non-mammalian species of lower neurophysiological sensitivity is not possible since they lack appropriate tissue physiology. We can't use embryos as the development of their various organs that are affected in human disease are not yet fully developed.

Also, all our targets are geared towards treating non-neonate humans and we need to ensure safety and efficacy at an equivalent timepoint.

Wherever possible, we will perform experiments in vitro or in non-protected species such as fruit flies or nematodes before progressing to mouse studies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Dedicated and trained staff will monitor and record animal status in all experiments using a dedicated electronic database. Parameters measured using a detailed monitoring regime will be recorded in the data base and on scoring sheets.

Whenever guidance materials are updated with recommended refinements, we will strive to include them into our procedures. We will regularly review literature and discuss possible refinements with peers and experts.

Where mice are housed in modified cages for testing it will be for the minimum time needed to gather data. Mice will be removed from test scenarios if suffering from an adverse stress reaction, or other unexpected adverse effects.

Animal rederivation and generation of new models will be performed with the help and support of the Genome Engineering team.

The support group at the Laboratory Animal Resource Unit has over 15 years' experience in refining surgical procedures, including the development of improved aseptic technique and rigorous analysis of surgery competency. We will continue to jointly refine skills and techniques to improve surgical outcomes.

Non-surgical methods of transferring embryos will be considered for mouse production. This will be assessed based on the ability to recover suitable numbers of live mice.

Prior to recovering strains for breeding, we will examine health observations previously published or recorded on a database that will allow us to accurately predict the onset of certain clinical signs of illness. We will then make notes on the database used by animal care staff so that they can quickly respond to welfare concerns. Furthermore, we will create a simple reference document listing all
strains that will be used on this licence, including description and if known time of onset for particular health concerns. This can be used by animal care staff as a guidance document.

There may be some experiments where we are able to administer drugs via the mouse diet (food or drinking water). Choosing this dose route is a refinement compared with other routes, such as by injection, where harms are known to be greater.

Access to and support from the imaging team will allow us to utilize specialized imaging techniques and might enable us to identify more subtle, yet undiscovered disease relevant phenotypes which will be used for subsequent monitoring of efficacy of novel therapies (performed under a different licence).

Technical refinements will be developed throughout the project and disseminated to other researchers and collaborators.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Diehl KH, et al., (2001) A good practice guide to the administration of substances and removal of blood, including routes and volumes. J Appl Toxicol, 21:15.
Workman W, et al., (2010) Guidelines for the welfare and use of animals in cancer research. Br J Cancer, 102, 1555.
LASA 2017 Guiding principles for preparing for and undertaking aseptic Surgery. A report by the LASA Education, Training and Ethics section. (E Lilley and M. Berdoy eds.)
Smith, et al., (2017) PREPARE: guidelines for planning animal research and testing
Ullman-Cullere, et.al, (1999) Body condition scoring: a rapid and accurate method for

assessing health status in mice. Lab Anim Sci., 49(3):319-23

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We maintain a close working relationship with all the staff, named persons at Laboratory Animal Resource Unit and with AWERB. This includes regular technical and training reviews. We will implement improvements according to new guidelines or recommendations as they are published for example on the NC3Rs website or disseminated from regional 3Rs symposia and meetings.

The PPL holder and delegates from the in vivo research team, will work together to adopt new developments in the area of 3Rs at the establishment the work will be carried out in. We will work closely with named persons and managers involved with promoting welfare, where possible, attending events promoting the 3Rs hosted locally and through institutions such as NC3Rs, LASA and the IAT. The International Society for Transgenic Technologies (ISTT) provides additional contacts and materials for keeping up to date with production technologies emphasizing the 3Rs.

We will also use our network of similar-minded investigators, including academic and industrial collaborators as well as patient advocacy groups.
Any new methodology, techniques or practical refinements will be tested against established techniques in small pilot studies and, where results are not compromised and animal welfare is improved, will be implemented accordingly.

57. Generation and validation of mouse models for the discovery of innovative antibodies with therapeutic potential

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Therapy, "Next-generation" antibodies, Cancer, Mouse model, Bispecific antibodies

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to generate and validate transgenic mouse models capable of producing innovative formats of human antibodies that can potentially be used as therapeutic drugs.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Over the past 30 years antibodies proved to be an extremely valuable therapeutic tool for the treatment of a variety of diseases, including many forms of cancer. Most of the

antibodies that have been approved so far for use in the clinic, were isolated from mice that are capable of producing human-like antibodies (a humanised mouse model). Despite the clinical success of therapeutic antibodies, there are still many pathologies that don't have an effective treatment and require the development of innovative type of drugs. "Conventional" antibodies (like the ones produced by healthy individuals) have limitations that restrict their use in some of the more advanced experimental therapies. For example, most drugs based on conventional antibodies struggle to reach targets located in the human brain because of their large size. Ongoing research has identified new, smaller antibodies that target diseased cells more effectively and penetrate better into specific types of human tissues. This is just an example of a growing family of "next-generation" antibodies that have the potential of revolutionizing the treatment of multiple diseases.

In this project we aim at generating and validating new mouse models that can produce "next- generation" antibodies. As we have done in the past with mouse models capable of producing fully human antibodies, our goal is to make them available to researchers around the world and expedite the development of much needed drugs. This would have a tremendous impact on the life of many patients that are waiting for new treatments to ameliorate or cure their conditions.

What outputs do you think you will see at the end of this project?

At the end of this project, the primary outcome will be the development and validation of multiple mouse models that have the capacity to produce human antibodies with special properties that makes them different from the "normal" or "canonical" antibodies produced by healthy individuals.

Such mouse models will be used to discover new drugs for a variety of conditions like cancer, autoimmune diseases (a disease where the immune system of an individual attacks and damages its own cells) and infectious diseases (a disease caused by different types of microorganisms like bacteria and viruses). These new mouse models will also allow to learn more about the biology of this new class of "next-generation" antibodies.

An example, is the development of the so called "common light chain" mice. This type of mouse will be engineered to produce human antibodies that are particularly suited to be assembled into a new class of therapeutic antibodies called "bi-specifics". As the name suggests, bi-specific antibodies can recognize two different targets at the same time compared to a "canonical" antibody that only recognises and binds a single target at the time. Antibodies engineered to recognize two targets at the same time have already showed very promising results for the treatment of multiple diseases, in particular cancer. Another goal of this project is the development of mice able to produce a novel class of antibodies called “VHH” antibodies or “nanobodies”. This novel class of antibodies is gaining a lot of attention among scientists and clinicians because their properties are particularly promising for targeting diseases that currently have limited therapeutic options.

Unfortunately, producing this type of innovative drugs is not straightforward due to the lack of efficient discovery platforms, but also due to the limited understanding of their biology. For this reason, fully validated and easily accessible discovery platforms for this type of innovative antibodies would be of crucial importance to support the rapid development of new drugs.

The mouse models generated in this project will hopefully become a very valuable platform

for the early discovery and early development of new therapeutic antibodies however they won't be used to produce the final product. The final therapeutic antibodies initially discovered through these mouse platforms will be produced using exclusively non-animal based technologies like for example bioreactors.

Who or what will benefit from these outputs, and how?

In the short term, mice generated in this study will allow us to learn more about the general biology of innovative classes of antibodies. This will be accomplished by our team during the validation process, but also by multiple research teams around the world with which we plan to share our mice. We also collaborate with academic groups that are involved in immunology and drug-discovery research and will benefit from the use of these new models for their studies. Whenever possible, the new findings will be published on scientific journals and the data shared with the wider scientific community. This will help disseminate data and additional information on these new types of antibodies and reduce the need of generating additional mouse models in the future (reduction).

On the medium and longer term, the goal is to create tools to allow the discovery of new, more efficient drugs, that can help improve patient's life. Mouse platforms for the discovery of innovative antibodies can significantly benefit a wide range of patients. For example, bi-specific antibodies, thanks to their ability to recognize two different targets simultaneously, can enhance the precision and effectiveness of cancer immunotherapy (a type of therapy that uses our own immune system to attack cancer cells).

This unique dual targeting capacity can lead to improved recruitment and activation of immune cells at the tumour site, offering a powerful strategy against various cancers.

Patients with neurological disorders like Alzheimer's and Parkinson's disease would also benefit from innovative antibodies. Single domain antibodies, for example, can reach the brain more easily than conventional antibodies due to their smaller size.

This makes them particularly suited for targeting this type of diseases. Preclinical studies with single domain antibodies in patients affected by Alzheimer's disease have shown a potential in preventing the onset of the disease an in improving cognitive functions.

Single domain antibodies have also shown an enhanced capacity in neutralising certain types of pathogens that can cause different types of infectious diseases. For example, specific types of single domain antibodies have shown promise in neutralising SARS-CoV-2, the virus responsible for COVID- 19, by binding to multiple sites on the virus, thereby preventing it from entering human cells. These initial findings can lead to the development of next-generation drugs against a range of widely common viruses and bacteria.

In this project, our minimum goal is to produce the following mouse models:
Common Light Chain (CLC) mouse for the discovery of bispecific antibodies. We are aiming to produce 3-4 different versions characterized by alternative genetic designs.

The goal is to generate a selection of transgenic mouse models that can be used for discovery projects against a wide range of human therapeutic targets.

Single Domain (SD) mouse for the discovery of "VHH antibodies" / "nanobodies". Also in this case, we are aiming to produce different versions (2-3 strains) characterized by

different genetic designs. The biology of VHH antibodies is still an area of active research and testing alternative designs will allow us to identify the best model(s) to support the discovery of this promising new type of therapeutic antibodies.

How will you look to maximise the outputs of this work?

Our company believes that our industry should compete on getting the best drugs to patients as quickly as possible by providing easier access to the best discovery platforms.

Our mission is to make medicines together, by working collaboratively with the global scientific community. For this reason, our goal is to establish and validate platforms that can be widely used by academic groups, small biotech and big pharma that are involved in therapeutic antibody discovery.

Humanised mouse models that our team developed in previous projects are now deployed by hundreds of discovery teams around the world and, as of 2024, more than 12 antibodies discovered from such platforms already entered clinical studies and are tested in patients. The goal for this project is to replicate the success that we achieved in the past by developing and distributing new platforms that can support the discovery on "next-generation" antibody-based drugs.

In the longer-term, when possible, key findings will be made available to other scientists through publications in scientific journals for the benefit of the wider scientific community.

This will include positive as well as negative outcomes. During this project, we will be testing different alternative designs to evaluate which specific configuration is more effective in supporting the development of non-canonical antibodies. Inevitably, some designs will prove to be better than others and sharing such information will inform other groups involved in similar projects and help avoid duplications and additional animal work (reduction).

Species and numbers of animals expected to be used.

- Mice: The maximum number of mice that we expect to use over a period of 5 years is 10000.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our project is divided in two major goals: 1) generation of new transgenic mouse models that can produce "non-canonical" antibodies with therapeutic potential and 2) validation and characterization of the newly generated mouse models.

The generation of new transgenic mouse models will be performed using a well characterised procedure that uses mouse Embryonic Stem Cells (mESC). Mouse ES cells are cell lines that are already available in our lab and can be propagated and manipulated

in vitro to introduce the desired genetic modifications that are investigated in this study.

Once the mES cells are successfully modified, they get injected into an early-stage embryo called blastocyst (this is also performed *in vitro*).

The blastocysts containing the injected mES cells can then be transferred into a recipient female mouse where the embryos can complete their normal development and produce transgenic pups. The genetic modifications need to be introduced into early-stage embryonic cells because they are easy to manipulate and have the unique property of being able to generate all the different tissues and organs of a fully developed mouse. This unique property is called "totipotency".

All the remaining steps of the work performed in this project, including the validation will be performed in adult mice. Breeding of mice will involve all stages of development as part of the normal life cycle of a mouse.

We chose mice as our animal model because their genetics are well understood, and current technologies enable the specific genetic modifications we need for this study. This wouldn't be possible (or it would be much more difficult) in other animal models. Mice have already proven to be an ideal model for discovery of clinical antibodies in previous studies, and we believe they will be particularly suitable for achieving the objectives described in this project.

Typically, what will be done to an animal used in your project?

The major procedures involved in the generation of new genetically altered mice are 2 minimally invasive surgical procedures. A small number of male mice will be vasectomized to render them sterile. This procedure only causes transient discomfort and has no long-term effects on the welfare of the mice. Non-surgical embryo transfer is the preferred method to implant the manipulated embryos into recipient females. Also in this case, the procedure is minimally invasive, and it only causes a transient mild discomfort.

During the validation process, mice will receive injections of different types of compounds. This is done to induce an immune response in the mice and evaluate their capacity of producing certain types of antibodies. This approach is very similar to vaccination in human patients and it might include multiple boosts to achieve an optimal immune response. The substances injected are not expected to cause any harm or cause the development of any type of disease in the mice. When needed, small samples of blood will be collected from peripheral veins to test the production of antibodies or to test other clinically relevant parameters.

Injections will be performed by highly trained technicians with the appropriate equipment and are expected to only cause transient discomfort with no long-lasting effects.

The following routes for injections will be used during the different procedure performed in this project:

Subcutaneous
Intravenous
Intradermal
Intraperitoneal

Intramuscular

Before the start of an immunization study, mice might be microchipped for identification purposes. Microchips are about the size of a grain of rice and are implanted under the skin of the mice using a special type of syringe. A combination of local anaesthetic cream and general anaesthetic is normally used to minimize discomfort.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice generated in this project, are not expected to develop any type of disease or pathology. They are expected to be healthy and behave like unmodified mice. Some of the genetically modified mice might have mild forms of immunodeficiency (conditions where the body's defence system is not fully functional) but that won't be associated with any harmful effect. All mice will be kept in pathogen-free facilities to avoid any potential risk of infection. All the substances injected into the experimental animals are non-toxic and not expected to cause pain, suffering, distress or lasting harm.

The only time when the mice might experience mild discomfort is during procedures such as injections or small surgeries. Animals subjected to these small surgery procedures will be treated with the most appropriate anaesthetic regimen and be provided with pain-killer medications if needed. The nature of the discomfort is expected to be of transient nature (less than 24 hours) and never exceed a moderate severity.

The administration of substances will be performed through different routes of injection that should cause no more than momentary pain.

Some immunisations will be performed mixing the antigen of interest with an adjuvant. An adjuvant is a substance that is added to help enhance the body's immune response to the antigen of interest. In simple terms, it acts like a booster, making the immunisation procedure more effective. A particular type of adjuvant called FCA (Freund's Complete Adjuvant) may be associated with small, non-painful nodules at the site of injection or in worse cases, in local tissue necrosis and ulcerations. FCA will be used no more than once and when used, mice will be monitored closely to ensure that any potential adverse reactions are identified promptly.

Insertion of microchips under the skin of mice, can cause localized inflammation and swelling that normally disappear within 24 hours. In rare cases, the body may form a granuloma, which is a small area of inflammation, around the microchip. This might persist for a few days post insertion. In very rare cases, if proper aseptic techniques are not followed, infection can be observed at the site of the microchip insertion. This can lead to localized swelling, redness, and in severe cases, abscess formation.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice Sub-threshold: 34.56%
Mild: 51.84%

Moderate: 13.60%
Severe: 0%

What will happen to animals used in this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Therapeutic antibodies can be identified using two different approaches: *in vitro* and *in vivo*. "*In vitro*" refers to experiments or procedures that are performed outside of a living organism, usually in a controlled laboratory environment. The most common *in vitro* methods used for antibody discovery are display technologies or computer-generated algorithms like artificial intelligence or machine learning.

On the contrary, "*in vivo*" refers to experiments or procedures that are performed within a living organism, such as a transgenic mouse.

Unfortunately, there is no *in vitro* system currently available that, on its own, can be used to generate appropriately modified antibodies with the diversity, specificity and properties that the *in vivo* models offer.

Which non-animal alternatives did you consider for use in this project?

Display technologies, like phage display, are powerful tools for discovering new antibodies, but they have limitations that mean they are not always sufficient on their own, to guarantee finding the best therapeutic antibodies. This is particularly true in the case of more sophisticated "next-generation" antibodies. We carefully consider all possible *in vitro* discovery platforms that can replace or reduce the use of *in vivo* models. We have a full team of scientists specialised in display technologies (phage, yeast and mammalian) and we often achieve the best results by combining these with *in vivo* platforms. Our team keeps improving display technologies implementing all the technical advancements that become available and also by applying what we learn from the use of animal models. In the long term, the goal is to be able to reduce as much as possible, and even replace animal work whenever is not strictly needed.

Artificial Intelligence (AI) and Machine Learning (ML) are also promising technologies that are already used by our scientists to facilitate the discovery of new therapeutic antibodies. Both technologies (AI and ML) are very helpful in complementing or facilitating the use of both *in vitro* and *in vivo* platforms but, at the current state of development, it's unrealistic that new therapeutic antibodies can be discovered with an *in silico*-only approach. As of 2024, no drug has been approved that was generated exclusively by AI or other *in silico* methods.

Why were they not suitable?

There are multiple reasons that limit the use of display technologies on their own. Below are some examples:

Artificial biological context
Display technologies often occur in a test tube or on the surface of a virus, which doesn't fully replicate the complex environment inside an animal body. Antibodies identified through display technologies might not work as effectively in real-life biological conditions because they haven't been selected in a living system with all its complexities. Phage display bypasses the natural immune system's selection mechanisms, which consider how antibodies interact with other parts of the immune system, such as T cells and various signalling molecules. As a result, antibodies identified from *in vitro* approaches have less chances to become functional drugs.

Lack of functionality and diversity.
While display technologies can identify antibodies that bind tightly to a target, this high affinity doesn't always translate into effective therapeutic action. For an antibody to be therapeutically useful, it needs to not only bind to its target but also induce the desired biological effect, such as blocking a receptor or inducing cell death.

The human immune system has a remarkable capacity of generating an astonishing number of antibodies. Considering all the different mechanisms deployed by the immune system to generate antibodies, it is estimated that the human body can produce on the order of 12^{12} to 10^{15} different antibody specificities! This incredible diversity can be replicated in mouse models (which our group achieved in the past) but it's much more challenging to achieve the same diversity in an *in vitro* system.

Even with the most modern technologies, it is hard for a phage display library to go over 10^{11} antibodies. This is multiple orders of magnitude lower than an *in vivo* system.

Specificity issues
Antibodies identified through display technologies might bind to unintended targets (off-target binding), potentially causing side effects. The immune system of a mouse or human has a quality control system that negatively select the antibodies with low affinity against the specific target or for any antibody that targets (and damage) the body itself (auto-reactive antibodies). This means that in general, antibodies isolated from *in vivo* platforms have a better safety profile while antibodies isolated from display technologies require a more comprehensive testing to ensure specificity and safety.

Developability
A major bottleneck in translating experimental antibodies into clinical antibodies is their developability (propensity to become a commercial drug). Antibodies identified through phage display can encounter several developability problems that need to be addressed before they can become effective therapeutic agents. These challenges include issues related, for example, to their physical stability or aggregation. On the contrary, antibodies isolated from *in vivo* platforms have been already selected by the immune system not only for their binding and functional properties but also for their stability and lack of aggregation.

Affinity vs. Functional Maturation
While additional rounds of biopanning can increase the affinity of phage library candidates, this process focuses primarily on binding affinity, often at the expense of other critical factors, such as antibody stability, specificity, or effector function. In vivo systems allow for a more holistic approach to antibody maturation, where the functional relevance of an antibody (e.g., its ability to trigger immune cell engagement, cross the blood-brain barrier, or neutralize a pathogen) is co-selected alongside affinity. This is particularly important for non-canonical antibodies, such as bispecific and single-domain antibodies, where function often depends on more complex interactions than simple high-affinity binding.

In conclusion, *in vitro* methods for antibody discovery are very powerful but on their own are limited by a multitude of issues that undermines their ability of isolating antibodies with clinical properties. The combination of *in vitro* and *in vivo* approaches gives the best chances of success and for this reason the development of animal platforms is of paramount importance.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimation is based on previous experience on similar research projects and on the type of goals that we plan to achieve in this project. Our research team has successfully worked on similar projects where transgenic mice capable of producing human antibodies were developed and validated. This helped us estimate the number of mice that will be required to generate a minimum of 5 new transgenic models and perform the validation and characterization of the newly developed strains.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

During the experimental design of our validation experiments we make sure to select the correct number of animals. The goal is always to use the smallest possible number of animals that allows to address our scientific objectives. We often use online resources to help us selecting the most appropriate cohorts size like for example, the Experimental Design Assistant (EDA) from the NC3R webpage (https://www.nc3rs.org.uk/our-portfolio/experimental-design-assistant-eda). When planning experimental animal work we make extensive use of the PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines (https://norecopa.no/prepare).

We also use data collected from previous experiments to help guide us in the design of similar studies. We have access to a team of experienced bioinformaticians and statisticians that we consult when we plan *in vivo* studies to make sure the typical variation observed in our own previous experiments is taken into account to calculate minimum number of animals to be used whilst ensuring that the results are statistically significant.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Different measures will be adopted in this project to optimise the number of animals used: For the generation of new genetically altered mice we will use mouse ES cells that have been previously characterized for their ability to produce high percentage chimeras (mice where both modified and unmodified cells and tissues are present at the same time) that have high potential to transmit their genetic modifications to the offspring (germline transmission). This will reduce the number of breeding steps required and prevents the generation of surplus animals.

Breeding of transgenic strains will be carefully planned and monitored to avoid overproducing mice that are not required for the purpose of this project.

At the end of each validation study all relevant tissues will be collected and cryopreserved (stored at very low temperatures) even if they are not needed at the time of the experiment. Additional data might be generated from these tissues at a later stage preventing the use of additional mice.

Wildtype (non-transgenic) animals that have been overproduced or that are the result of breeding experiments will be made available to other research groups in the facility whenever possible and following consultation with the facility veterinarian.

When possible, mice will be challenged with multiple antigens simultaneously to reduce the overall number of mice used. This will only be considered when the presence of multiple antigens doesn't interfere with the scientific outcome of the experiment and when potential toxicity effects have been completely ruled out.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The generation of new genetically altered mice will be performed by introducing the desired modifications into ES cell lines with well-defined *in vitro* procedures. The engineered ES cells will then be used to produce early-stage embryos containing the desired modifications. All these steps don't cause any type of pain since they are performed on cell lines or on very premature embryos. To achieve full development of the embryos, they will then be implanted into recipient females. This procedure, whenever possible, will be achieved through a non-surgical procedure that is minimally invasive and causes very mild distress. In some circumstances, a surgical approach might be chosen and in this case the mice will be treated with an appropriate anaesthetic regimen in combination with pre- and post-operative analgesic treatment if required. All procedures

will be performed by experienced and trained operators who will ensure best practises are followed. This way, the distress and the risk of complications will be reduced to the very minimum.

Adult mice will be tested for their capacity of mounting an immune response and produce the types of antibodies that are investigated in this project. This will be done by injecting the mice through standard routes (e.g. subcutaneous injections) in a way that replicates immunizations in human patients. The type of substances injected, the route of injection and the frequency of injection is optimized to achieve a good scientific output and, at the same time, limit as much as possible the distress of the animals.

Immunizations are not expected to cause any long-lasting pain nor cause diseases to the mice. The handling of the mice and the injection itself is expected to produce a transient mild discomfort that will resolve within a few hours. During procedures, mice will be closely monitored to identify any early sign of distress or pain. In very rare cases where unexpected severe side effects are reported, the mice will be humanely killed.

Why can’t you use animals that are less sentient?

To achieve the overall goal of this project it is not possible to use animals that are less sentient than mice for several reasons:

Mice have an immune system that is more similar to humans compared to less sentient animals like invertebrates. This similarity includes the structure and function of immune cells and antibodies, making mice a more suitable model for studying immune responses and developing therapeutic antibodies.

Mice can generate a diverse array of antibodies through different complex processes called "V(D)J recombination", "somatic hypermutation", and "class switching".

Humans and mice share the same mechanisms for generating antibody diversity, enabling mice models to accurately replicate the human immune response. Less sentient animals often lack these complex immune mechanisms.

Mice can be genetically engineered to express human immune components (humanization), which allows for the discovery of fully human antibodies for therapeutic use. This process is not feasible with less sentient animals like invertebrates.

We can’t use embryos or very young animals as their immune system is immature and doesn’t respond to antigenic stimulation in the way mature animals do.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice will be kept in specific pathogen-free facilities and will be handled by highly trained animal care staff to ensure that any type of distress is minimised. Animals will be given environmental enrichments including plastic houses and cardboard tubes and, when possible, will be group housed to reduce isolation stress. The mice will be handled in such a way as to reduce stress (for example using cupped hands or cardboard tubes). If mice are imported from another facility, they will be allowed to acclimate prior to any procedures.

The route of injection used in this project, including intraperitoneal and subcutaneous injections, have all been shown to cause no adverse effects whilst inducing effective antibody responses in most cases. Animal suffering will be minimised by limiting the numbers of boosts and the duration of the experiment. The levels of antibodies present in the circulating blood (serum titres) will be used to determine the number of boosts and therefore the scientific endpoints. All substances used for mice immunizations will be tested to ensure they are sterile, pure and that endotoxins (an immunogenic contaminant derived from bacterial cells) are not present. Injections will be performed by trained technicians to minimise stress and discomfort.

We will develop a standardised check-list scoring sheet to monitor the health status of the mice after immunisations to ensure that no adverse reactions are overlooked.

The score card will include several parameters including the following:
General appearance
weight loss, coat condition, posture, dehydration. The mouse grimace scale (https://nc3rs.org.uk/3rs- resource-library/grimace-scales/grimace-scale-mouse) will be used to assess post-procedural pain as part of the clinical assessment.

Behaviour
activity level, grooming, social interaction, eating and drinking.

Additional parameters
breathing rate and effort, tremors or seizures, diarrhoea, abdominal distension, red or swollen eyes, nasal discharge, sensitivity to touch, vocalizations.

Very few, if any, animals will undergo surgical procedures. However, if they do, then pre and post- operative pain relief will be given.

For immunizations, small pilot studies will be conducted to ensure that the methods used minimise harm while meeting the study objectives.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Best practice guidance from different sources will be followed. This will include:
-PREPARE guidelines (http://journals.sagepub.com/doi/full/10.1177/0023677217724823), very helpful to assist the planning of animal research.

ARRIVE (Animal Research: Reporting of In Vivo Experiments) Guidelines (https://arriveguidelines.org/), provides a comprehensive framework for reporting scientific results and ensure studies are conducted rigorously and transparently.
Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 (UK Home Office)
NC3Rs best practice guidelines (https://nc3rs.org.uk/)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed about advances in the 3Rs by checking regularly the NC3Rs webpage and by signing up to alerts and newsletters from relevant organizations like for

example NC3Rs, AWI (Animal Welfare Institute), and FRAME (Fund for the Replacement of Animals in Medical Experiments). The University Biomedical Services (UBS) runs an in-house 3R's enquire list to which I am planning to become a member.

We will participate in online courses and webinars that provide updates and training on the latest 3Rs methodologies and technologies and have regular interactions with experts in our network like NACWOs and NVSs.

Whenever new developments in the 3Rs become available we will do our best to implement them straight away in our experimental project.

58. Preventing cancer initiation and metastasis

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, Metastasis, Prevention, Therapy, Metabolism

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to further understand the regulatory mechanisms of cancer initiation and metastasis, to identify and develop therapies to prevent cancer and cancer progression, and to translate laboratory evidence to guide and facilitate the design and conduct of clinical trials.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

In the UK, survival of patients with organ-confined cancer has steadily improved with advances in diagnosis and therapy, however, the fact remains that ~167,000 people are likely to die from cancer annually, particularly attributable to metastatic disease (cancer spreading or secondary tumour). This is because cancer is often diagnosed late and metastasis may begin before the primary tumour is discovered. Therefore, finding novel

interventions to prevent the initiation of both primary and secondary cancer is equally, if not more, important than exploring effective therapies for established cases.

In our previous research, we have established means to track rare, individual stem cell like cancer cells responsible for initiating cancer and their metastasis (secondary cancer sites), and identified some of their targetable unique features. More importantly, through several successfully funded research programs, we demonstrated that existing well-established drugs or diet-derived compounds (e.g. Cancer Research UK sponsored COLO-PREVENT study), and other lifestyle factors particularly exercise (Prostate Cancer UK sponsored Research Innovation Award), can prevent cancer initiation, progression and metastasis.

Our research is now at a critical stage, ready to translate this basic knowledge into clinical applications and benefit the patients at the earliest opportunity.

In this project, we will use appropriate animals to accurately mimic the different stages of cancer progression and continue to investigate key factors controlling the fate cancer stem cells. This will not only complement our understanding in this important area, but also potentially identify novel targets for cancer prevent and treatment.

We will further assess the antitumour effects of diet-derived compounds, repurposed drugs, and lifestyle changes such as exercise, in order to gather clinically translatable information, including treatment dosage, frequency, and duration. This information will guide future patient based clinical trials. The effects of these treatments may also be assessed in combination with the usage of other standard of care practices for enhanced protection.

Finally, we will also examine the effects of these treatment strategies on healthy animals to explore and establish biological markers, such as blood tests, for their efficacy and potential side effects.

What outputs do you think you will see at the end of this project?

The work conducted under this project will lead to the identification of safe diet-derived agents, repurposed drugs, and lifestyle interventions with the potential to prevent cancer in humans and/or improve the treatment of cancer. The results will be used to optimise the design and monitoring of clinical trials to test the therapies in volunteers and patients. In addition, better understanding of mechanisms regulating cancer initiation and metastasis will lead to novel treatment targets and high impact scientific publications.

Who or what will benefit from these outputs, and how?

We expect that the information gained will not only help to rapidly trial promising approaches in patient cohorts but also facilitate the identification of which people are most likely to benefit from taking a particular therapy to prevent cancer so trials can be focused specifically on these people. Ultimately, the successful discovery of effective therapies would help prevent an immense amount of human suffering and save precious health service resources.

How will you look to maximise the outputs of this work?

We will disseminate our research outputs both within and beyond the academic

community. Academically, this will be achieved via publishing our research in high quality peer reviewed journals and presenting research in national and internationally conferences. Generated data will be stored in both local archives and openly accessible data base, e.g. Gene Expression Omnibus (GEO), and made available on request to other researchers around the world. We will expand our collaborations with basic scientists and clinicians locally and nationally/internationally at other research institutes. We will distribute our new mouse models and tissues collected from the mice freely to other researchers.

Non-academically, the primary goal is to enhance active engagement with partners from industrial sectors and to exploit the commercial impact of our research outputs. This will be achieved by knowledge exchange and jointly exploring the potential discovery of new anti-cancer drugs or repurposing of existing drugs based on our findings. We will also ensure that our research is effectively communicated to the public, particularly regarding lifestyle and dietary studies. To achieve this, we will work closely with the Media Release group within our institute and provide updated research outcome for press release.

Species and numbers of animals expected to be used

- Mice: 3700

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Since this proposal focuses on the initiation and metastasis of cancer cells, which is an integrated process involving multiple cellular interactions, we will mainly use well established mouse models of cancer, including mesothelioma, colorectal, breast, prostate, pancreatic cancer etc. The age of mice at the start of each study will be 5-10 weeks old when tumour take rates are high and disease (tumour growth and bone disease) variability is relatively low according to our previous experience.

Typically, what will be done to an animal used in your project?

During the studies, mice will be injected with tumour cells via various routes, such as under the skin (subcutaneously), into organs (orthotopically), or systemically (intracardiac or intravascular), depending on the specific research question been addressed. Disease progression (tumour growth and metastasis) will be monitored by imaging methods.

Interventions (exercise and/or drugs) will be administered and effects assessed at different stages of disease progression by imaging (e.g. bioluminescence and micro-CT).

What are the expected impacts and/or adverse effects for the animals during your project?

Potential adverse effects for tumour growth and metastasis are weight loss (>15%), hypothermia, enlarged lymph glands, ulceration, hind limb paralysis/weakness, poor locomotion, hunching and/or tremors or convulsions or disturbed breathing, and other

signs of pain. If any of these signs are observed animals will be killed immediately.

Where surgery is required, we will minimize wound breakdown and infection using appropriate aseptic technique and suitable training. Pain will be controlled using effective anaesthesia and analgesics and will be assessed using the principles of the grimace scale for mice throughout each study.

No adverse effects are expected for evaluation of pharmaceutical agents or exercise regimens other than the possibility of transient discomfort where injectable routes, or gavage are used and following repeated doses.

Mice may be killed at specific time points depending on the research question been addressed or euthanised at the end of the experiments.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severities for all tumour models, for the evaluation of pharmaceutical agents and/or exercise, and for breeding and maintenance of genetically modified animals will be moderate.

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Since this proposal focuses on the initiation of both primary and secondary tumours, which is an integrated process involving multiple cellular, tissue and organ interactions, the use of animals is essential to achieve the proposed objectives.

There are no surrogate models available that encompass all the potential interactions present in vivo. In addition, the use of live animals is also essential to assess the effects of promising preventive therapies in terms of efficacy and tolerability/toxicity on all major systems in vivo. Experiments in mice are required to take into account the integrated effects of metabolism, distribution and elimination and also to study interactions between diet/metabolic factors and preventive therapies.

Which non-animal alternatives did you consider for use in this project?

All pharmacological agents/drugs will be firstly tested in vitro in cell lines and state-of-the-art ex vivo cultures of intact pieces of tissue and 3-dimensional cell structures, by

measuring the changes in proliferation and programmed death of cancer cells, as well as their ability to migrate and invade new tissues. This will enable us to identify the most promising agents, at their predicted optimal doses, and then subsequently advance to in vivo testing in mouse models. For exercise regimens, we will firstly evaluate and optimised in established computational models (in silico) and then verify the results in living animals (in vivo).

Why were they not suitable?

Initiation of cancer and metastasis is a complex process involving physical and signal interactions among a variety of molecules, cells, tissues and organs. Therefore, it is not possible to accurately mimic these complex conditions in vitro, particularly when many factors involved are still unknown. Moreover, in vitro testing neither provides complete mechanistic data nor accounts for any systemic effects in animals (e.g. diet/metabolic factors). Therefore, we must rely on well-designed animal studies to carry out the proposed studies and achieve translational benefits for patients at the earliest opportunities.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Our previous experience of conducting the types of experiments planned in this project ensures that the minimum numbers of mice are used to generate statistically significant results for each type of study. Conventionally, 12-14 animals/treatment group were required to provide enough statistical power to detect significant differences in tumour burden in studies using mouse model bearing human tumours.

However, our recent experience of quantitative analyses of previous xenograft studies and further statistical analysis and power calculation showed that with greater refinement, and 9 mice/group will provide enough statistical power to generate robust data. Considering a 90% tumour engraftment rate, we have decided to use 10 mice per experimental group, in order to balance between reducing the number of mice needed and achieve sufficient statistical power for all proposed animal studies. This is also a widely used group size believed to be statistically robust and scientifically and clinically relevant within the oncology community. Base on this group size and predicted number of studies we expect to perform under each protocol annually (20-30 studies), we estimate the number of animals to be used is 3700.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Statistical advice will be sought throughout the programme of work (from our local Statistical Advisory Service) to specifically support animal experimenters and by using the NC3Rs Experimental Design Assistant (EDA) to determine sample size.

We will use groups of animals that are very closely matched in age, sex, health status, and stress level, to minimise variability. We will use new technology that enable us to adopt more efficient experiment deigns (e.g. factorial design) reduce number of animals. For example, we will use in vivo micro-CT to carry out longitudinal factorial studies instead of multiple cross-sectional studies so that significantly reduced number of animals will be used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will continuously work closely with biostatisticians and ensure the results obtained will be subjected to detailed statistical analyses to obtain the maximum amount of information possible from each experiment. We will keep abreast with the advance of scientific literature and instructions from regulatory bodies relating to reduction of usage of animals.

We will always perform pilot studies before major studies to ensure all methods are completely established. We have devised a breeding strategy that minimises variation in tumour development in our genetic models of cancer and therefore leads to more consistent results, which minimises animal numbers. We will make tissue available to other researchers and ensure the maximum usage of tissues.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Our primary aim is to develop strategies to prevent cancer. To achieve this, we focus on the early stages of tumour development. We use specially bred mice with specific genetic changes that mimic the initial steps of cancer formation. For example, our colorectal cancer model involves removing the Apc gene, which is commonly mutated in most human cases of this cancer type. This makes the mouse model closely resemble the human condition.

When suitable genetically modified mice are unavailable, we use mice inoculated with cancer cells as models. These include implanting human cancer cells into mice with weakened immune systems to test treatments, or transplanting mouse tumours into other mice with fully functioning immune systems.

We use two main methods to introduce tumours in these models. For primary cancer studies, we place cancer cells under the skin or directly into specific organs. For metastasis studies, we introduce cancer cells into the bloodstream through methods such as injecting them into the heart or blood vessels.

These approaches are standard and widely used for studying cancer and metastasis in animals.

Our team has extensive experience in these techniques, which are well-documented and reliable. In our work, we consistently achieve a high success rate in tumour growth (>90%), as shown in our previous studies. We have gained extensive experience in the management of these animal models and have learnt to quickly identify adverse effects and spot early signs of distress, so animals are culled before disease progresses, further minimizing pain, suffering, distress and lasting harm.

Why can't you use animals that are less sentient?

Some less-sentient species, such as fruit fly and zebrafish, are very useful models to carry out mechanistic studies or studies on certain steps during the tumorigenic processes (e.g. tumour cell extravasation). However, our studies are focusing on the prevention and treatments on cancer initiation, growth, spreading and relapse, so requires a whole organism model closely resemble human patients. Therefore, we need to use mammals and mice are the least sentient mammalian species that we can use.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To further minimise welfare costs to the animals, we will adopt, but not limited to, the following measures:

Ensure all procedures follow the best practice guidelines (e.g. LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery, NCRI Guidelines for the welfare and use of animals in cancer research, and the PREPARE guidelines),
Ensure small pilot studies are always performed to identify optimal treatment regimens (e.g. dosage, frequency etc) before to be used in larger scale studies, Plan experiments to minimize unnecessary harm, distress, and pain to animals (e.g. not let tumours grow to a size causing the animal to become significantly unwell),
Mice will be monitored and assessed regularly for signs of being unwell, pain, suffering, or distress, using the Morton & Griffiths assessment. Earlier humane endpoint will always be adopted to minimise harm and suffering,
Invasive surgeries will be performed under general anaesthesia and analgesic will be given to manage pain. These procedures are only to be carried out by competent and experienced staffs,
Invasive procedures will be carried out on a cadaver under the supervision of our NVS before carrying out a recovery surgery,
Maximise welfare in housing and care, e.g. providing enrichment in housing, refining handling technique (use of tunnels), and pre-screening tumour cells to be injected for potential pathogens.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The NC3Rs website contains a vast range of guidance for best practice across the spectrum of animal research activities. (https://www.nc3rs.org.uk/3rs-resources).

CAAT-Europe also disseminates information about developments within 3Rs in the

European network, which NC3Rs is part of. For the design and planning of animal experiments, we will follow the PREPARE guidelines. For all procedures involved in cancer initiation and metastasis, we will follow the NCRI Guidelines for the welfare and use of animals in cancer research. All procedures involving surgeries will be performed under sterile conditions and following the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will attend relevant trainings, workshops and conferences nationally and internationally, and read scientific literature to stay informed of new advances. We will also regularly discuss the 3Rs with staff in the animal facility, as well as consult the NC3Rs and CAAT-Europe online resources.

59. Regulation of adaptive immune and autoimmune responses

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Adaptive immunity, Antibody maturation, B cells, Somatic hypermutation, Inflammatory arthritis

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The goal of this project is to understand how B cells (a type of white blood cell) develop stronger and more specific antibodies during immune responses. This process, known as antibody maturation, helps our bodies produce antibodies that effectively fight off infections and respond to vaccines. This happens through a process called somatic hypermutation, where the genes that produce antibodies are intentionally mutated to create a diverse range of antibodies. Our research primarily focuses on this process. However, somatic hypermutation can also lead to the production of harmful antibodies that attack the body's own tissues, causing autoimmune diseases like inflammatory arthritis. Therefore, another long-term aim of our research is to find ways to control somatic hypermutation to reduce the symptoms of these autoimmune diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Robust adaptive immune responses are necessary for a health and wellbeing. But they are also responsible for triggering autoimmune diseases and cancers due to their mutagenic nature. Adaptive immune responses also weaken with age. Therefore, understanding how adaptive immunity works is necessary in order to design effective therapies that can boost or suppress the these pathways.

What outputs do you think you will see at the end of this project?

These studies will create new knowledge about the mechanisms underlying somatic hypermutation, the latter process being the principle means by which the immune system generates antibodies with high affinity for target antigens present on pathogens and vaccines. We expect our studies to result in multiple publications.

Importantly, by studying the role of various factors involved in somatic hypermutation, we will be able to determine whether they can be used as potential drug targets to alleviate symptoms of autoimmune disease which will be further explored using the excellent resources and expertise at KCL for translational research.

Who or what will benefit from these outputs, and how?

In the short term, our work will address the hypothesis that autoimmune disease symptoms can be alleviated by targeting somatic hypermutation pathways. If promising, the results could fuel further research in this direction with the potential of long-term translational outputs. Specifically, there is a need for alternative strategies to alleviate pain and suffering in patients with autoimmune disease, since available therapies do not work in all cases and often cause relapses. We hope that suppressing somatic hypermutation by targeted therapy can mitigate some of the symptoms in patients and, therefore, act as an adjunct therapy in caring for individuals with autoimmune disease.

Upon publication, the results will contribute to the research in other laboratories across the scientific community. The projects may drive new collaborations, both locally and internationally, that could have major long-term benefits for all sides by creating new opportunities for collaborative projects and grant proposals aimed at further expanding research into the mechanisms of adaptive immunity.

How will you look to maximise the outputs of this work?

We expect these studies to yield new knowledge of the molecular mechanisms of adaptive immune responses. We also expect to forge collaborations with colleagues at KCL and elsewhere for specific aims, for example, the analysis of collagen-induced arthritis, bioinformatics analyses and single cell RNA-sequencing analysis.

Irrespective of the results, that is, even if the results are negative, we expect to publish our findings in peer-reviewed journals.

If our results indicate that loss of somatic hypermutation can reduce the severity of arthritis in mice, it would suggest a possible translational potential which we will pursue using the vast expertise in translational and clinical research available at KCL and in the UK as a whole, both within academia and via industry collaborations.

Species and numbers of animals expected to be used

- Mice: 34,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adaptive immunity is a complex process involving interactions between different cell types and tissue microenvironments. This complexity cannot be recreated in vitro and hence requires the use of live animals, especially mice, whose immune systems are similar to humans and where the tools for genetic manipulation and immune cell analyses are available and firmly established. We use adult mice, both wild-type and genetically modified, that are at least 6 weeks old because our experiments require that their immune systems are fully developed and responsive to challenges.

Typically, what will be done to an animal used in your project?

Some mice (both wild-type and genetically altered) will be killed without any procedures performed on them. The spleens or these mice will be used to isolate and culture immune cells in vitro to study the effect of the deleted genes on specific processes such as antibody production and gene expression.

Some mice (both wild-type and genetically altered) will be injected with vaccines (e.g. hapten-protein conjugates or protein in alum adjuvant) or cells (e.g. sheep red blood cells) to trigger an adaptive immune response without inflammation or disease. This will typically be done once or, occasionally, twice, and 10-14 days later, they will be killed and their spleens, lymph nodes and Peyer's patches harvested. These tissues will be used to study the composition and properties of immune cell populations by flow cytometry and genomics. In some cases, sections of these tissues will be frozen for histological analyses.

Occasionally, blood samples will be taken to measure the levels of antibodies in the serum.

Some mice (both wild-type and genetically altered) will be subjected to radiation to ablate their bone marrow followed by reconstitution of the bone marrow by injection of bone marrow from other mice. This reconstitution procedure usually takes 6-8 weeks after which will be immunized with vaccines and processed as described in point 2 above.

Some mice (both wild-type and genetically altered) will be used to model inflammatory arthritis by injecting them once or twice with type II collagen in complete Freund's adjuvant (collagen-induced arthritis). Mice will be kept alive for a maximum of 8 weeks after onset of symptoms after which they will be killed by a Schedule 1 method. Before this time-point, any mice showing pain and suffering beyond the defined humane end-points, will be killed immediately by a schedule 1 method.

What are the expected impacts and/or adverse effects for the animals during your project?

Procedures using non-inflammatory immunization and adoptive transfer of cells are only

expected to cause minimal, short-term (few hours to a day) discomfort at the injection site.

Some animals may be injected with tamoxifen to induce gene deletion, which cause weight loss between 5-15%. More infrequently (<5% of animals), increased weight loss can occur along with reduced mobility, hunched posture, shivering and piloerection.

Bone marrow reconstitution can occasionally cause infection due to immunosuppression (~2%), acute bleeding due to cytopenia (~2%), diarrhoea and dehydration (~5%).

Following irradiation and transplantation, animals will often show signs of malaise manifested by a hunched posture, reduced activity and/or staring coat, evidence of weight loss relative to sex/age matched animals of a similar genetic background (up to 15% weight loss, lasting approximately 2 weeks).

For the inflammatory arthritis procedure, joint stiffness and some degree of pain and reduced mobility is expected across the duration of the experiment. Due to complete Freund's adjuvant, most mice will develop redness, roughening and loss of fur at the site of injection. Mice will normally be killed within two weeks of arthritis symptom onset. In a small proportion of mice (<5%), ulceration may develop at the site of injection. Irrespective of these time limits, animals will be monitored at least three times a week (e.g. by inspecting injection sites, weighing them and scoring their paw swelling) and culled according to the humane end points.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We expect ~80% of mice to undergo mild procedures and ~20% to undergo moderate procedure.

The mild procedures are breeding, immunization with non-inflammatory vaccines and cells, and adoptive transfer experiments.

The moderate procedures are tamoxifen injection to induce gene deletion, bone marrow reconstitution and the collagen-induced arthritis protocol.

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The adaptive immune response occurs in a complex environment within lymphoid tissues

consisting of a diversity of immune and non-immune cells in varying developmental states.

Moreover, interactions between these cells, which are crucial for proper immune responses, occur within discrete anatomically polarized structures called germinal centers, where cycles of somatic hypermutation and antigen affinity-based selection occur in distinct zones over several weeks. Such complexity cannot be recapitulated in vitro and, consequently, animal models are indispensable for elucidating the mechanisms governing adaptive immunity.

Which non-animal alternatives did you consider for use in this project?

Certain aspects of the adaptive immune response can be recapitulated to some degree (though certainly not comprehensively) in B cell lines in vitro. For example, the Ramos human B cell line has been used to study somatic hypermutation to some degree because it undergoes the early stages of the hypermutation process, albeit much less efficiently than germinal center B cells in vivo. It is nonetheless a useful discovery tool and we have used it to perform genetic screens to identify potential candidate factors regulating somatic hypermutation which we validated (or will validate as part of this project) using conditional-knockout mouse models.

A murine B cell line, CH12, has also been widely used to study antibody isotype class switching, but only to the IgA isotype, which is a major limitation. However, it remains a good tool to identify new factors regulating class switch recombination and studying mechanistic aspects.

We routinely use such cell lines for mechanistic studies, where possible, and will continue to use them to address certain questions that these systems are adequate for.

Why were they not suitable?

It is important to note that the in vitro culture systems can complement, but never replace, the mouse models. systems. As mentioned above, the highly specialized and complex germinal center environment, where somatic hypermutation and affinity selection occur, cannot be recreated in vitro which precludes the in vitro analysis of most of the major processes involved in adaptive immunity.

Since many defects in somatic hypermutation can result from changes in germinal center size, cellular composition of germinal centers (including interaction with helper T cells) and architectural polarity of germinal centers, mouse models are essential to comprehensively understand. Finally, since the cell lines are derived from transformed cells or tumours, one needs in vivo mouse genetic analysis to ensure that the factors, pathways and mechanisms identified in cell lines are relevant physiologically. These severe limitations of in vitro systems necessitate the use of live mice.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot

studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Experiments will generally focus on a single primary scientific objective, unless exploring additional objectives within the same experimental groups can yield valuable information, thereby reducing the number of mice required. Typically, genetically modified mice (e.g. conditional knockout and heterozygous genotypes) will be compared to suitable wild-type controls, with or without prior immunization, resulting in one or two experimental groups and one control group.

When applicable, control animals may be shared among multiple experimental groups, minimizing the overall mouse usage. For all experiments, relevant tissues will be utilized and/or stored for analysis and future studies, minimizing the need for experiment repetition.

To minimize variability and ensure the production of high-quality, reproducible, and reliable results, the following measures will be implemented:

(1) using animals of the same background and similar age;
(2) employing animals of both genders unless specific gender effects are present; and (3) housing animals in the same location throughout the experiment. Standard protocols will be followed for all experimentation and data analysis.

Initial group size determination will be based on resource equation-determined pilot studies, investigating the primary outcome necessary to statistically achieve the scientific aim. Furthermore, insights from previous work conducted by our team and collaborators (as applicable) will contribute to determining an appropriate group size, aiming to achieve the scientific objective with minimal animal usage. Calculations typically indicate a need for group sizes between 6-10 to meet our scientific aims.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We utilized the NC3Rs' guidance on experimental design and their Experimental Design Assistant (EDA) to structure our experimental plans. Each protocol has been optimally designed to maximize research output while minimizing the use of animals required to meet our scientific objectives. As data is generated, we will consistently employ the NC3Rs' experimental design guidance and EDA, along with updated statistical calculations, to ensure the utilization of the least number of animals necessary to fulfill our scientific goals.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will closely monitor and optimize the breeding process to ensure the most effective utilization of animals. Pilot studies will be conducted in cases where there is limited or no preliminary data, with the aim of to establish a solid scientific foundation for future experiments. Upon the conclusion of the experiment, we will harvest as many tissues as feasible during postmortem procedures. If immediate analysis of the tissues is unnecessary, we will freeze them, making them accessible to other researchers exploring

similar questions.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

All protocols are ultimately aimed at studying the mechanisms of the adaptive immune responses in B cells in wild-type and genetically altered mice. The latter include conditional gene knockout mice (mice with floxed alleles crossed with lineage-specific Cre delete mice), which do not suffer from disease or illness since the gene deletion only occurs in specific lineages and, when using ERT2-cre, only upon injection with tamoxifen. We will also sometimes use mice expressing genetically modified antigen receptors, Cas9 and fluorescent reporters of gene expression or differentiation state, none of which shown any signs of distress or discomfort.

Adaptive immune responses will be studied following immunization e.g. hapten conjugates or cells (mild severity i.e. causing only minimal discomfort) or collagen in complete Freund’s adjuvant (CFA) (moderate severity causing pain and distress).

For collagen-CFA immunizations, a widely used model of rheumatoid arthritis, mice may be briefly restrained and/or briefly anesthetized for subcutaneous injection. For bone marrow reconstitution (also a moderate procedure), mice will be irradiated followed by intravenous (tail vein) injection of donor cells from another mouse.

All methodology has been refined over several years to use appropriate minimum group sizes, the lowest viable doses/volumes and limit the number of overall procedures. In each protocol, animals will undergo vigilant monitoring for various symptoms indicative of distress. Should any distress be identified, animals will be subjected to an intensified daily monitoring regimen. If distress levels reach pre-established endpoints, the animals will be humanely euthanized, following the procedures outlined in the protocols.

Why can’t you use animals that are less sentient?

Modeling human immune responses and diseases requires systems that replicate relevant aspects of human immunity. The laboratory mouse is the optimal choice given its close resemblance to human physiology and its invaluable contribution (spanning several decades of research) towards understanding the mechanisms of mammalian immunity. As a result, there is a vast body of literature, repositories of genetically modified sperm and embryos for genetic deletions and gene tagging, and optimized protocols for a host of immunological, histological and pathological analyses. We are unaware of any other less sentient model organism that can satisfy all these criteria.

The utilization of embryos or young animals is impractical, as their immature immune systems do not respond adequately to immune challenges, thereby failing to accurately model human adaptive immunity and autoimmune diseases.

Moreover, studying adaptive immune responses in anesthetized animals is not feasible as these experiments necessitate days or weeks of responses post-immunization to achieve the scientific objectives. However, mild, recovery anesthesia may be used briefly for the induction of inflammatory arthritis via subcutaneous injection of collagen.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Rigorous monitoring is conducted on all animals that undergo procedures, with those infected by inflammatory agents or chemicals assessed through weight checks, examinations, and assessments based on relevant distress symptoms and weight loss. In cases of distress, enhanced daily monitoring is promptly implemented.

Furthermore, if a procedure is known to heighten susceptibility to infection and potentially increase distress, animals undergo daily weighing and examination to ensure distress and severity remain within defined limits. If any animal experiences distress or severity beyond the defined thresholds in the protocol, humane euthanasia is promptly administered.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will adhere to the PREPARE guidelines for planning and executing experiments to ensure that the research is conducted in the most refined manner possible.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We subscribe to the 3R's newsletter, participate in local 3R's seminars, and attend presentations on the subject at conferences. Keeping abreast of updates on the 3R's website, we integrate new ideas and tools into our research whenever feasible.

60. A new calcium channel in cardio(vascular) disorders – A target to understand disease pathology

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Electrophysiology, Ion Channel, Cardiovascular, Calcium

Animal types	Life stages
Mice	Adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To determine the role of a newly identified calcium ion channel in cardiovascular function

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cardiovascular diseases (CVDs) are the leading cause of death globally. The development of novel, targeted treatment strategies for CVDs require new foundational knowledge of pathways that drive the pathophysiology of this group of diseases. A significant proportion of this program of work will investigate the involvement of a newly discovered ion channel in the heart and vascular system which we believe is upregulated and displays increased

activity in cardiovascular complications.

Cell culture systems, despite recent progress, cannot recapitulate the complexities of a whole system approach and are therefore inadequate to address all aims outlined in this project. In addition, computational approaches cannot recapitulate the unknowns of the circulatory system and its complex network of the heart and blood vessels. Currently there are no drugs or tools to manipulate the function of this channel. The genetically altered knock out mouse lacks the gene that codes for this protein and therefore provides a system to interrogate the function of this channel within the cardiovascular system. The mouse is a good model with established protocols for isolation of primary cells including cardiomyocytes and mesenteric and aortic vessels.

What outputs do you think you will see at the end of this project?

The primary potential benefit of this project relates to new knowledge about the role of a newly identified calcium release channel in controlling the beat-to-beat of the heart and also regulating blood pressure. These findings will advance our knowledge of how irregular activity of this channel can contribute to the generation of cardiovascular complications. The aim is to publish the findings in academic journals where the information is likely to be of interest to scientists interested in cardiovascular science and ion channel physiology.

There is also a secondary potential benefit which relates to the value of the results to clinicians, in particular cardiologists, and to the possibility that new molecular targets may be identified, for which new pharmaceutical products could be developed.

This detailed mechanistic study will provide much needed information regarding the physiological role of this new calcium channel and will highlight this protein as a new target in the treatment of heart failure. Key findings from this work will contribute to our understanding of cardiac diseases and help to pinpoint mechanisms that can be manipulated for therapeutic benefit.

Outputs will include new information that will be disseminated by scientific channels (publications, conference talks) and also to the via more accessible channels including social media, our research website, and also the University newsletter.

Who or what will benefit from these outputs, and how?

Cardiovascular diseases (CVDs) are the leading cause of death globally. The development of novel, targeted treatment strategies for CVDs require new foundational knowledge of pathways that drive the pathophysiology of this group of diseases. A significant proportion of this program of work will investigate the involvement of a newly discovered ion channel in the heart which we believe is upregulated and displays increased activity in cardiovascular complications.

In the short term the proposed work will provide much needed information regarding the physiological role of this new calcium channel and will highlight this protein as a new target in the treatment of cardiovascular complications. Key findings from this work will contribute to our understanding of cardiovascular diseases and help to pinpoint mechanisms that can be manipulated for therapeutic benefit.

Morbidity and mortality remain high for individuals with cardiovascular disease, and there is

a major need for the development of new therapeutic strategies.

Cardioprotective interventions hold great promise for diminishing the detrimental effects of myocardial ischaemic injury and heart failure. The expected value of this proposed work will therefore be of interest to cardiovascular scientists.

There is also a secondary potential benefit which relates to the value of the results to clinicians, in particular cardiologists, and to the possibility that new molecular targets may be identified, for which new pharmaceutical products could be developed. Given that our latest data show that knocking out the gene that codes for this protein protects against hypertrophy and heart dysfunction, the rationale is that in the long term if we understand the role of this protein in cardiovascular function and identify it as a new target in the failing heart, we may be able to develop pharmacological agents that can target this calcium channel to down regulate its activity. Such pharmacological agents could be given intravenously to patients at the early stages of heart failure, to prevent progression to chronic stages that are life threatening.

How will you look to maximise the outputs of this work?

Data will be made available following manuscript acceptance for publication. A data management plan (DMPonline) will be created at the start of the project. Publications will have annotated datasets in the supplementary files with links to raw data deposited in an appropriate repository and DOI assigned.

We will disseminate our data through preprints, journal publications and invited talks at research institutions and national and international meetings. Publication of new results will be followed by social media announcements as well as by press releases.

We will continue to work with our collaborators and seek to establish new ones to drive the research and reach the full potential of the work.

Species and numbers of animals expected to be used.

- Mice: 980 MG23 KO male and female mice

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The mouse is the species of choice for several reasons: first, its sufficient homology to humans makes it an excellent system to study the mechanisms underlying human disease. Indeed, our primary findings using mouse models and mouse tissue have underpinned the biology to enable us to make the first step towards human translation by looking at human genetics and moving into human stem cell technology. Second, the mouse is one of the most studied organisms, our data therefore will complement existing literature. Third, mice are good models for genetic manipulation. Currently there are no pharmacological tools to manipulate the function of this new calcium channel. The knock

out mouse, that lacks that gene that codes for this protein, therefore provides a system to interrogate the function of this protein in the cardiovascular system. Finally, the mouse is a good model with established protocols for isolation of primary cells including cardiomyocytes and mesenteric and aortic vessels.

We will use adult mice up to 12 months of age but typically between the ages of 12-24 weeks. This is the window used for most biological studies and so our data will comparable with historical data. We have extended the window from the previous application (16-20 weeks) to maximize usage of animals.

Typically, what will be done to an animal used in your project?

Breeding and maintenance of genetically altered mice by conventional breeding methods

- Tissue biopsy to determine genetic status by ear punch.
- Animals will be killed by a Schedule 1 method.
- Use of tissue with other groups (once animal has been killed) to maximise use of animal.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals produced under this protocol are not expected to exhibit any harmful phenotype.

Animals exhibiting any unexpected harmful phenotypes will be killed by schedule 1 methods. In the case of individual animals of particular scientific interest, we will submit a request to keep alive via ARSU mailbox.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Our experiments will involve the use heart and vascular tissue obtained from mice following the killing of animals via humane methods. The severity of the procedures conducted are expected to be subthreshold. The genetically modified mice are already established and currently housed at the institution. They show no obvious sign of any adverse effects. Severity is therefore graded in the sub- threshold or mild range.

What will happen to animals used in this project?

- Killed
- Kept alive at a licensed establishment for non-regulated purposes or possible reuse

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.
Why do you need to use animals to achieve the aim of your project?

Our project aim is to gain insights into the role of a new calcium channel in driving cardiovascular complications. Cardiovascular disease affects the heart and circulatory system. Cell culture systems, despite recent progress, cannot recapitulate the complexities of a whole system approach and are therefore inadequate to address all aims outlined in this project. In addition, computational approaches cannot recapitulate the unknowns of the circulatory system and its complex network of the heart and blood vessels. There remain too many unknowns for computer simulations to replace the use of animal tissue.

Which non-animal alternatives did you consider for use in this project?

We have recently begun to utilise human stem-cell derived cell culture models to investigate the basic function of this new ion channel. The gene that codes for this ion channel is included in genomic datasets looking at certain cardiovascular phenotypes and these data are available in public databases (Genebass) but this give us no information on the molecular pathways that are altered and result in pathology.

There are currently no data on the role of this new calcium channel using cardiac cell lines or IPSCs or organoids.

Why were they not suitable?

Human stem cells are electrically different from mature heart cells. Full testing of the role of this calcium channel in regulating heart beat and blood pressure can therefore only be validated using a genetically altered mouse model whereby the protein is lacking. There are no other mouse models available to investigate this protein.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Samples sizes were calculated using GPower (3.1.9.2). Effect sizes were determined with pilot and published data. Two-tailed power calculations were selected, where power was set to 0.8 with a statistical cut-off level of 0.05. Our preliminary and published material were used to determine the standard deviation of data. Both male and female mice will be used in the study. Numbers reported are intended to be the maximum number of animals required.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Experimental design will be planned utilizing the NC3Rs Experimental Design Assistant (EDA) which will be linked to publications and data-repositories. Based on previous experiments undertaken in my research group or based on published work by other groups

a standard t-test or ANOVA (for testing of multiple groups) are the significance tests of choice. We can access the assistance of a statistician to help plan appropriate group sizes for studies where there is variance in the effect size, providing advice on the planning and interpretation of experiments to achieve statistically meaningful outputs.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will follow current breeding strategies for maintaining colonies of laboratory mice set out by NC3Rs breeding and colony management advice.

Following identification of genetic status, animals produced under the authority of this project and not used in other regulated procedures may be supplied to other projects with authority to use genetically altered animals of this type.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Our gene of interest has no known adverse effects. The advantage of using a transgenic mouse model (where the protein of interest is lacking) is that it may give a more accurate result than using standard molecular methods which only reduce the amount of protein expressed. Such standard (knock-down) molecular methods have a low efficiency rate and the level of protein expression can be variable. The use of transgenic mice will therefore reduce variability and produce better scientific results.

This will minimise the number of animals required over the lifetime of the project.

We will only use schedule 1 killing methods on genetically altered mice.

Animals exhibiting any unexpected harmful phenotypes will be killed by schedule 1 methods.

Why can’t you use animals that are less sentient?

There are currently no in vitro models/organoids able to model the cardiac dynamics in a whole mammalian system. Mouse share the excitation contraction coupling processes of human myocytes that make them a suitable model species (although they also have well-recognised limitations). Adult mouse and human cardiac myocytes are structurally similar, they have well organised t-tubule networks coupled to the sarcoplasmic reticulum dyad structures, together these features are hallmarks of heart cell maturity and are important for cell function. Embryonic mice and species that are less sentient lack these

morphological and physiological similarities.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will only use schedule 1 killing methods on genetically altered mice. This will be carried out by trained and competent staff.

During the lifetime of the study should any animals display any unexpected harmful phenotypes they will be killed by Schedule 1 methods. In the case of individual animals of particular scientific interest, we will submit a request to keep alive via ARSU mailbox.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Research will be designed to conform to recognised best practice following guidance developed by the NC3Rs. Developments in best practice will be reviewed via publications and other resources accessible through the NC3Rs website.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will regularly check the NC3R website for current advice and guidance.

The institutions Home Office Liaison Officer and animal unit team are excellent at communicating relevant training opportunities about advances in the 3Rs.

The institution also runs an annual 3Rs event.

In collaboration with the animal unit at the institution, such advances may be implemented into the project.

61. Breeding and Aging of Genetically Altered or Natural Mutant Animals

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Breeding, Maintenance, Aging, GAA

Animal types	Life stages	
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult, Aged animal Rats	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To provide a demand-matched supply of genetically altered rodents of high health status and defined genetic quality.

To maintain animals including genetically modified animals in their old age.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

As, we gain greater knowledge of the human genome, the demand for genetically altered animals continues. New emergent technologies including genome- editing tools has led to an increase in more sophisticated models. This licence will allow the breeding, maintenance and aging of mouse and rat models for further use in other programmes of work or for tissue collection in order to study gene function that will provide fundamental

knowledge about normal biology, ageing and disease.

At our establishment, the use of genetically altered animals underpins a wide range of scientific projects and contributes greatly to scientists understanding of many biological processes including:

- endocrine function
- cardiovascular physiology
- cancer
- bone disease
- inflammatory processes
- hearing loss
- Neurological disorders
- immune responses

Due to the increasing lifespan age of humans globally, there is a greater need to study and understand aged animals in research to investigate frailty and other diseases (e.g. atherosclerosis, dementia) and disorders of old age linked to the examples mentioned above.

What outputs do you think you will see at the end of this project?

This is a service licence to support a full range of research at the university which underpin a wide range of scientific projects and contributes greatly to scientists understanding of many biological processes. A mouse passport is generated for all mouse strains. This includes information about background, breeding and phenotypic effects.

Who or what will benefit from these outputs, and how?

This licence will allow researchers at our establishment, who do not themselves hold a project licence, to breed and age GM and WT animals for tissue collection, avoiding the necessity for multiple project licences for tissue supply alone. The will also enable the multiple scientists to study and understand of a wide range of biological processes including:

- endocrine function
- cardiovascular physiology
- cancer
- bone disease
- inflammatory processes
- hearing loss
- Neurological disorders
- immune responses

It enables me to allocate dedicated experienced technicians to manage colonies, which brings benefits for breeding, genetic management and prompt identification of phenotypes, all of which minimises overbreeding and improves animal welfare.

How will you look to maximise the outputs of this work?

Mice and rats will be issued as requested and pairs set up to replace the issued animals. If larger numbers were to be requested, then the breeding would be increased accordingly.

This method is designed to keep stocks to a minimum.

Wherever possible standing orders will be encouraged so supply can be accurately controlled and stocks kept to a minimum. All strains entering or leaving the unit are accompanied with a mouse passport which gives in depth detail about the strain and identifies any phenotypic effects, like changes in behaviour, the gene modification may have.

Species and numbers of animals expected to be used.

- Mice: 5100
- Rats: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice and rats of adult breeding age are required for breeding production. All ages of animals from embryo to aged animals may be used for tissue collection.

Aged mice are needed for the supply of aged tissue. Due to the increasing lifespan age of humans globally, there is an increasing need for ageing animals in research to investigate frailty and diseases and disorders of old age.

Typically, what will be done to an animal used in your project?

Female animals will be mated under protocol 1, step 1 up to 7 times over the course of their breeding lifespan. Males of the correct genotype may be mated on numerous occasions. Offspring will be genotyped typically using an ear notch step 2, however, the other methods may be used to determine the correct genotype. Offspring of the correct genotype may be moved to a separate protocol (protocol 2) on this project to be housed long term to provide aged mice for studies, or supplied to other project licences. Animals that have reach the end of their useful breeding life will be humanely killed to prevent harms from breeding when too old.

Aged animals will be maintained and monitored until they have reached up to 20 months age or welfare becomes compromised, at which point they will be humanely killed for tissue.

What are the expected impacts and/or adverse effects for the animals during your project?

Mouse and Rat Breeding:

Animals bred and maintained under this project are not expected to exhibit a phenotype that will exceed mild severity. However there will be careful monitoring for possible side effects/phenotypes:

We expect the genetically altered lines bred under this project to exhibit similar rates of pre-weaning losses and adult mortality to the equivalent wild-type mice.

Aging Mice:
Mice may display general ageing phenotypes including one or more of the following issues: skin/hair defects, loss of body weight, behavioural changes, gait and mobility issues, and respiratory difficulties. The incidence increases with age with the likeliness of more than two of the above issues low for animals younger than 18 months, and higher for animals age 18-20 months. Our "traffic light" scoring system for the expected signs of ageing will be used to monitor how animals age and minimise their suffering by including additional care, or humanely killing mice which are suffering from conditions of old age.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice - 5% moderate, 25% mild, 70% sub-threshold.
Rats - 20% mild, 80% sub-threshold.

Aged animal on protocol 2 may develop mild dysfunction in their old age. Although the severity of the discomfort is mild, it may be there for prolonged periods of time and the accumulative effects would then become a moderate severity.

What will happen to animals used in this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Because of the nature of the licence there are no alternatives other than to use live animals to generate more live animals.

Which non-animal alternatives did you consider for use in this project?

N/A

Why were they not suitable?
N/A

Reduction

Explain how the numbers of animals for this project were determined. Describe

steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Numbers are based on previous Home Office returns. The use of aged animals is a new protocol, so numbers required is estimated.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Breeding programmes can be tightly controlled in accordance with the needs of the end user. Setting pre-determined targets minimises over production of animals which would otherwise be surplus to requirements.

Good working knowledge of the lines being actively bred allows optimum use of those animals in research programmes allows future requirements to be anticipated and minimises duplicate requests.

Mice will only be kept and aged if a suitable justification is provided. Welfare of the animals will be closely monitored, with consultation with the NVS. Any animals exceeding moderate severity will be culled.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Only well-characterised lines and strains of genetically altered rodents with phenotypes not exceeding mild severity will be bred and maintained.

Breeding programmes can be tightly controlled in accordance with the needs of the end user. Setting pre-determined targets minimises over production of animals which would otherwise be surplus to requirements.

Good working knowledge of the lines being actively bred allows optimum use of those animals in research programmes and also allows future requirements to be anticipated and minimises duplicate requests.

In some instances where unexpectedly surplus stock has been identified by the colony manager animals may be offered to the scientific research groups within the University to limit waste and better utilise available resources. The project licence holder will ensure that potential availability of surplus animals is not exploited by an end user as an alternative to holding his/her own project licence.
Specific justification for the use and ageing of the animals will be required from the end user on a case- by-case basis and will be considered by the Biological Services Unit.

Tissues collected from aged mice may be shared by multiple end users.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice and Rats will be used under the project for breeding. Tissue biopsy may be used to genotype GA animals but will seek to use a more refined method, such as saliva or hair.

Why can’t you use animals that are less sentient?

This PPL is based on researcher requirements. Mature animals are required to produce further animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The techniques are established and the limitations and potential problems are known.

The animals will be cared for by dedicated animal technologists who have the expertise and skills required in the breeding and ageing of animals.

Experienced animal technicians are able to asses any welfare problems that may occur at an early stage and are able to determine appropriate end points in consultation with the NACWO and NVS.

The transgenic colonies will be maintained to the same high standards as all other animals in the unit, with health screening to FELASA standards.

Animals that are known to exceed mild severity will not be bred on this licence.
All aged animals will be monitored daily, any welfare issues will be discussed with the NVS so as to ensure they do not exceed moderate severity.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Home Office
Breeding of Genetically Altered Animals Assessment Framework

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The NC3Rs website will be consulted to ensure up to date 3Rs advances are implemented.

Our local AWERB also has a 3Rs sub-committee where the 3Rs are discussed and new

approaches disseminated to researchers in-house.
Attending conferences and networking with other breeders will also ensure best practice is maintained.

62. Circadian and nuclear receptor regulation of inflammation and energy metabolism

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Inflammation, Metabolism, Circadian, Therapy, Nuclear receptors

Animal types	Life stages
Mice	Neonate, Juvenile, Adult, Pregnant adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to further our understanding of the interactions between the circadian system, metabolism and inflammation, to discover new ways to help people living with inflammatory and energy metabolic diseases.

Specifically, we will investigate the body’s internal clock controls energy use and inflammation under normal conditions, and how this control changes when the body is under stress such as obesity, environment challenges (cold or comfortable temperatures), or inflammation. We will further investigate how changes in energy levels like obesity, or inflammation, affect the circadian system. In this way we can understand why human diseases so often change in severity through the day, why changes to our rhythmic organisation of life (like shift work), increase the risk of inflammatory diseases, and obesity, and why diseases change the function of the circadian system, resulting in long-term damage, and affecting response to treatment.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

There is a massive burden of disease with people living longer, and limited ways in which we can help. Inflammation plays a critical role in most human diseases, but safe effective treatments remain an unmet and urgent clinical need. The immune system is a potent regulator of cellular and systemic energy metabolism so a better understanding of their interactions is crucial. All aspects of human physiology are mapped onto a 24h rhythm, orchestrated by an endogenous circadian timing system, and its circadian dysfunction is a contributory factor to many pathological conditions like sleep disorders, cancer, metabolic syndrome or inflammatory disease.

We urgently need new, innovative ways to help diagnose, and treat people with inflammatory, and metabolic diseases, eg diabetes, metabolic syndrome, obesity, arthritis, lung disease.

What outputs do you think you will see at the end of this project?

- New targets for drug development.
- New ways to use existing drugs and therapies in a better, more effective, and safer manner. Discoveries to increase scientific knowledge in the field of inflammatory and metabolic diseases.
- New approaches to diagnosing people.

Who or what will benefit from these outputs, and how?

In the short term, we expect to discover and describe new targets and new mechanisms involved in these diseases that will benefit the scientific community and pharmaceutical industry: the outputs will increase the scientific knowledge allowing the development of new technology, new therapeutic targets and solve/answer key questions.

In the long term, we expect our outputs will contribute to the development of more efficient diagnosis combined with improved or new treatments, having a clear positive impact on patients with inflammation or metabolic disorders but also will have an impact on future generations with risk factors for these diseases.

How will you look to maximise the outputs of this work?

The outputs of this project will be presented in national and international conferences and meetings, to disseminate the new discoveries and to generate discussion with colleagues and experts on the field. This will bring new ideas, different approaches and new collaborations, including sharing data and tissues.
In addition, positive and negative results will be published in open-access scientific journals, making available the data to the scientific community.

Species and numbers of animals expected to be used.

- Mice: 14000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our work requires a mammalian model that is similar in physiology to the human, therefore we use the mouse which is an ideal model for this purpose. Mice experience many of the same disease as humans, specifically for metabolic and inflammatory diseases. In addition, in mice it is easy to manipulate the central and peripheral circadian clock to be able to reflect that of humans. The mouse and its genetic map has been fully sequenced; this provides us an excellent and easy genetic tool to generate genetically altered models which greatly help us to identify molecular contributors to these pathologies.

Mice are typically used both in juvenile and adult life stages (~3 months - ~6 months for metabolic/circadian procedures). We use this stage because after three months mice have completed the maturational growth for most biological processes and structures. Mice up to 12 months old can be used for the breeding protocol.

Typically, what will be done to an animal used in your project?

Typical exposures include altering the light conditions, altered feeding schedule, some injections, and a blood sample. Some mice may be temporary single housed for some of these investigations, for example when we measure energy metabolism.

We will use mainly models of mice with some specific genetic alteration in genes involved in circadian rhythm, inflammation or metabolic disorders. Some of our experiments will use wild type and/or genetic modified mice, that may be fed with normal diet or with some specific diet, for example high fat diet to generate diet induced obese models.

Some animals will undergo alteration of the light/dark cycle (or normal 12h/12h light/dark cycle) in specific cabinets to evaluate the physiological response to circadian alteration.

Metabolic parameters can be measured in the same cabinets such as energy consumption, locomotor activity or food intake. Also, for precise measurements of fat and/or lean mass, or water content, the animals may undergo an MRI scan.

Some animals can also undergo metabolic tests like insulin or glucose tolerance test, to evaluate their metabolic response. Some animals may also have their blood taken, but these are not expected to cause any harm that is more than transient and mild.

Some animals will undergo thermal challenges, through exposure to cold or normal/higher temperature. The first one involves placing their cage at a low temperature for a defined period of time, with frequent monitoring. The purpose is for animals to generate heat to measure how fat is used to generate heat in the brown adipose tissue. This is a normal physiological response in mammals. The second one, involves placing animals at 34C (normal conditions) for a defined period of time with frequent monitoring, to evaluate how

animals regulate their temperature and metabolism under not challenged conditions.

Some animals will undergo surgical interventions under general anaesthesia; for example to implant small devices under the skin (to monitor body temperature) or to implant a small device that allows the administration of substances continuously.

Mice are given pain relief, just like human patients, after a minor operation.

At the end of the experiments all animals are humanely killed, and tissues harvested to be analysed post-mortem.

What are the expected impacts and/or adverse effects for the animals during your project?

The animals will experience disturbance from altered lighting and food, but they adapt quickly and easily. Regular cages where animals are housed are used in the specific cabinets to altered light/dark conditions or to measure metabolic parameters, to minimize any kind of disturbance altering the environment.

Transient pain will be suffered with injections, plus distress from handling. Getting blood samples can be distressing, but for a short period of time. The volume extracted will be also small.

Mice will have minor surgery for example to implant a device under the skin that can release a substance slowly (avoiding daily injection). They are expected to recover quickly and will be given painkillers and post-operative care just like people recovering in hospital.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Sub threshold 50%
Mild 30%
Moderate 20%

What will happen to animals used in this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We are mainly studying the interplay between three key systems in the body: immune,

circadian and metabolic system. We need to use animal models to understand the interactions between them.

Specifically, we study the body clock and its role in making our bodies work well and how alterations in circadian rhythm can interfere with our immune system and our metabolic response. This involves several organs with many different cell types interacting and communicating with each other, creating a complex interplay that is not possible to replicate *in vitro.*

Which non-animal alternatives did you consider for use in this project?

We consider, and use, human cohorts and small numbers of human volunteers who we study intensively in our clinical research facility, but the number and possible interventions are limited.

In addition, we also contemplate the possibility of using human organoids to study organ homeostasis *in vitro,* however to recapitulate *in vitro* the highly complex structure of in vivo tissue and the interrelationships between body systems remains challenging and cannot fully simulate the tissue microenvironment observed *in vivo.*

We complement our studies in animals with immortal cell lines but their functional levels are frequently different from those found in primary cells and some important functions are missed.

Why were they not suitable?

We study liver, muscle, fat and brain. It is not possible to obtain tissue from all these organs from human volunteers. We also investigate gene function by deleting genes, again not possible in people. We test new compounds which might lead to new drugs, but which are not possible or safe to be tested in people, but they can be used in animals.

Organoid systems and immortal cell lines are interesting and useful for some molecular approaches but they do not replicate the complexity of the interplay between circadian, immune and metabolic systems.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We've used our annual return of procedures data to estimate the number of animals that we will need to use for breeding. Data on previous experiments has been used to calculate the minimum number of animals needed for the protocols. Calculations typically show that we need group sizes of 6-8 to achieve the quality of results we need.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We work closely with statisticians to provide information on animal experiment design to justify the animal numbers proposed. We based these calculations on the previous work in our lab using similar protocols, and where not available we turn to the literature, and to colleagues, and if the protocol is quite new we may conduct an initial pilot to estimate the number of animals required.

We will use free online tools like the Experimental Design Assistant (EDA) from the NC3Rs, to design and determine the appropriate sample size for our experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Breeding colonies will be managed in line with the best practice guidelines. Particular attention will be paid to genetic stability and good breeding performance. Data from breeding animals are readily available from the in-house database and will be used to make decisions on future breeding animals and to assist in maintaining a suitable colony size to ensure only those animals needed for experiments are produced.

Cryopreservation of colonies not required in the short term will be considered.

We sometimes use pilot studies, or base our designs on previous work in our lab, and in the labs of colleagues to ensure only those animals needed for experiments are produced.

We always share tissues, and make the maximal use of archived human and animals tissues to avoid unnecessary breeding, and animal use.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Our studies will focus on the use of laboratory mice. This species offers unparalleled opportunities for investigations of the underlying genetic mechanisms involved in circadian timing, immune and metabolism function, an essential pre-requisite for targeting of pathways with novel drugs and therapies. We have established and validated colonies of genetically altered mice that lack either a functional clock, or receptors/proteins involved in metabolism, in different cell types. These are excellent tools for addressing our key questions and will provide insight into clock control of immuno- inflammation, while minimising pain, suffering, distress and lasting harm.

The protocols employed are well established and designed not to induce severe responses or suffering in animals. For instance, in our studies of acute inflammatory responses, we are specifically interested in determining time-of-day and genetic differences and will therefore employ low doses of inflammatory substances, which induce a sub-maximal response. Similarly, our studies of energy metabolic stress include diet alterations (for example high fat feeding protocols which induce obesity), but which are well-tolerated by the mice. We will make use of environmental manipulation by manipulating light cycles to induce circadian disruption, or by altering the temperature of the cage. This will be transitory and we know that mice adapt quite fast.

We will make use of small, implanted devices to track core body temperature, and from the same device, we can also measure other physiological parameters.

Similarly, a small device to avoid several injections may be implanted. All minor surgical interventions are well established and cause only minor and transient pain.

In general, we can challenge the animal by making changes to the environment, but we have tools to monitor the response of the animal and terminate the exposure at the point of physiological adaptation but before decompensation and suffering is observed.

Why can’t you use animals that are less sentient?

All the conditions that we study are developed during adult age. To make our research translational as much as we can, we have to perform the procedures in adult mice mainly (after 2-3 months old when the development is fully completed and before 6 months to avoid the presence of age-related changes); and live animals to see the whole physiology response of the organism.

Mice experience many of the same diseases as humans and have the same types of organs and bodily systems, which makes them excellent models for human disease.

Around 95% of the genes that code for proteins are identical in humans and mice. Species that are less sentient such as zebrafish or flies cannot be used as they don't have a fully functional metabolic/immune system. They are not mammals, and although there are genes that are equivalent to human genes, they don't all act in the same way. In addition, some drugs that work in flies don't work or are not safe in humans so the translation of our studies could be highly compromised.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Improving husbandry standards to allow for more species-specific behaviour. Conditioning of animals for parts of the experimental procedure, for example through positive reinforcement.

Animals are closely monitored after the administration of any new substances (e.g. check of alterations in body weight and behaviour).

Animals receive appropriate medication following surgery. Improving anaesthesia and analgesia protocols.

Alternative application techniques based on the study and substance (e.g. oral administration instead intraperitoneal injections for oily solutions).

Updating and adaptation to the protocols (e.g. reduction of fasting period to the minimal for glucose or insulin tests).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines: to include all the information describing animal research in publications, to be sure they are reported in enough detail and can be reproducible and reliable for the researchers and reviewers.

Laboratory Animal Science Association (LASA): to ensure the best use of the most appropriate animal models and derived materials (organs, tissues and cellular components) in our research.

NC3R's: to develop and use 3Rs technologies and approaches appropriately.
UKRI Guidance for applications involving animals: to help and support the scientific strength of grant applications.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have subscribed to the NC3R's newsletter and regularly check the website for new advances in the 3R's. We attend internal welfare and 3R themed meetings at the establishment. Our Named Information Officer also disseminates new material in relation to the 3R's.

Specific to our field, we work with national, and international networks studying animal models of the circadian system, and actively participate in 3Rs innovation.

Examples include the UK Clock club, Society for Research into Biological Rhythms, and European Biological Rhythm Society.

63. Developing RNA Gene Therapies to treat neurodegenerative Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Motor Neuron Disease, Gene therapy, Dementia, Amyotrophic Lateral Sclerosis, Adeno Associate Virus

Animal types	Life stages
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult, Aged animal

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This research project focuses on creating new treatments for degenerative genetic brain and spinal cord diseases, such as motor neurone disease (MND) and frontotemporal dementia (FTD), by targeting genetic causes like the C9orf72, FUS and TDP-43 gene mutation. By using a modified, non- infectious virus (AAV) to deliver genetic RNA material, the approach aims to deactivate faulty gene segments and restore normal gene function, offering hope for effective therapies for diseases that currently lack cures.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could

be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Undertaking this work is crucial because there are currently no effective drugs for many genetic diseases, particularly those linked to aging and related neurodegeneration, such as motor neurone disease (MND) and frontotemporal dementia (FTD). In the UK, MND affects approximately 5,000 people at any given time, while globally, it impacts around 140,000 individuals. FTD is estimated to affect tens of thousands in the UK and hundreds of thousands worldwide, making it a leading cause of early-onset dementia. With the ageing population, the number of elderly individuals suffering from these diseases is rising, creating immense challenges for families and caregivers. The financial burden is also significant, with the global cost of dementia exceeding $1 trillion annually and similar pressures on the UK's healthcare system. Developing effective treatments is, therefore, critical to improving the quality of life for patients and reducing the overall burden on families and healthcare providers. This project aims to meet this need by creating innovative gene therapy approaches that can modify disease progression in preclinical models, potentially translating into effective therapies for patients worldwide, including those in the UK.

What outputs do you think you will see at the end of this project?

The output from this project will be novel therapies for incurable neurological degenerative disorders that can be delivered to human trials. The AAV is used in clinical Moreover, we are developing a novel platform of non-coding and coding RNA transgene and adeno associate virus (AAV)capsids for non- invasive delivery that can be applied to the broad population of degenerative diseases, which could effectively slow down the disease progress by using the RNA therapeutic platform.

Who or what will benefit from these outputs, and how?

The primary aim of this project is to collect pre-clinical data on prospective gene therapy treatments for a range of currently un-treatable neurodegenerative disorders, with the goal of moving promising therapeutics into clinical trials. Hence both researchers in the field and patients with these devastating disorders, as well as their families, carers and clinicians are likely to benefit from the outputs generated in this project.

How will you look to maximise the outputs of this work?

The RNA gene therapy project has been approved by LifeArc/MNDA, and its primary goal is to deliver effective therapies to patients. To facilitate this work, a milestone-based study plan has been established to maximise the potential benefits of prospective therapies and newly developed methods.

The successful candidates of new RNA elements will be filed for patent application, and the fundamental knowledge will be shared with the community which will accelerate research in the field of gene regulation and controlling the unwanted toxic genes. All of the discoveries will be published in journals and shared with collaborators and any relevant researchers who want to further develop the system for academic research.

To share the new findings, we will establish a supporting group where we can disseminate the relevant information through accurate data analysis and assessment.

Species and numbers of animals expected to be used.

- Mice: 10500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project aims to test the efficacy of novel RNA gene therapies for neurodegenerative diseases such as motor neuron disease, dementia and diabetic neurodegenerative disease. These diseases affect the brain and spinal cord as we age, and in order to determine if the therapies have an impact on the progression of the disease, we will study them in aged mice (12-24 month). The mouse was selected as it possesses a cerebral cortex, which is only found in mammals and is preferentially affected in many neurodegenerative diseases. The mice used in this project will have genetic alterations that are known to cause neurodegenerative diseases in humans, allowing us to see if the treatments are effective at reducing or preventing the development of these disease features. For instance, the rapid decline in motor behavior observed in TDP43 and FUS transgenic mice, which serve as a model of motor neuron disease, enable us to access the efficacy of various treatments using a well-established and disease relevant readout.

Typically, what will be done to an animal used in your project?

This project is divided into two main parts. The first aims to improve how we can target disease affected cells, while the second is focused on modifying cell function to prevent or reduce the disease process. For the first aim, normal mice will receive a brain injection of viral particles that have been engineered to deliver a possible disease treatment in order to develop the most effective delivery system. A portion of these mice will be kept up to 2 years of age for tissue collection.

For the second aim, initially normal mice will receive an injection either into the brain or body that contains a virus to deliver the potential new treatment (typically a gene or RNA) to check for any evidence of unexpected/unwanted side effects. These mice will undergo behavioural tests to assess brain and spinal cord function, including movement tests.

Finally, mice that model the neurodegenerative disease of interest will receive the potential therapy or placebo via the same route (brain or body injection) and again will undergo behavioural tests to determine the effectiveness of the treatment. All mice will be aged up to a maximum of 2 years and will be culled for tissue collection at the end of the study period.

What are the expected impacts and/or adverse effects for the animals during your project?

The animals in this project may experience some discomfort following brain injections, but this is not expected to have impact more than 24 hours effects. The degenerative disease models to be used may develop mobility or memory issues as they age. While memory issues may not greatly impact mouse welfare, mobility problems may cause mild to moderate difficulties in movement, but these are not associated with pain. If the motor impairments worsen, steps will be taken to ensure the animals can successfully feed, such as providing moistened food at floor level. If there is a decline in general health, the animal will be culled. In addition, old animals may develop age-related issues such as benign tumours or hair loss, and if they cause significant pain, discomfort or distress, the animal will be culled.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

80% moderate and 20 % mild.

What will happen to animals used in this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This project's goal is to evaluate the efficacy of novel RNA gene therapies for degenerative diseases by using live animal models to determine if they can slow or halt the advancement of the disease's clinical features, which is crucial to establish their effectiveness in humans. Early screens for new therapies will be conducted in cells to confirm that these treatments are viable, and do not result in any significant negative effects. However, only by conducting in vivo studies in a functional nervous system can we ensure we target our specific cells of choice, and fully assess the potential value of these new therapeutic approaches to target clinical symptoms of disease.

Which non-animal alternatives did you consider for use in this project?

This project also utilizes in vitro methods, such as growing various cells, including induced pluripotent stem cells (iPSC), in a cell culture plate and CNS slice cultures, which are thin slices of the CNS that are grown in a cell culture plate. These methods provide a way to study cells and tissues in a controlled environment and can offer insights into the underlying mechanisms of degenerative diseases and the potential effectiveness of treatments without using live animals.

Why were they not suitable?

A key objective of this project is to determine if the potential new AAV gene therapy delivery and treatments can alleviate movement and memory impairments associated with degenerative disorders such as motor neuron disease and dementia.

This can only be assessed in living animals. Additionally, the study aims to understand how the treatments may affect the progression of the disease in the specific area of the brain and spinal cord over time. Currently, non-animal models cannot fully replicate the complexity of the AAV delivery route from non-invasive systemic injection due to barriers such as the blood-brain barrier (BBB)preventing penetration to the brain.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals used in each protocol of this project has been carefully calculated based on the specific aims and objectives of the project.

For the assessment of phenotypic changes in genetically modified mouse lines or other disease models without current robust phenotyping data, numbers have been determined based on our experience with similar pre-existing mouse lines, and the estimated number of unique genes or gene variants we plan to investigate.

For the validation and testing of new viral delivery systems, numbers have been estimated based on the total required for validation of each system and the number of different variants we anticipate testing.

For therapy assessment, numbers have been estimated based on the total required for validation of a single therapy or therapeutic target, as well as the number of different disease models and therapies we plan to test over the course of the project.

Furthermore, we have also taken into consideration the number of animals needed to maintain the different lines of genetically modified mice throughout the project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

During the design phase of each experiment, extensive research was conducted to identify available mouse lines that would be suitable for the study and to ensure access to all relevant information regarding expected outcomes in these animals.

Every effort was made to choose lines that would enable easy measurement of the impact of the therapies on the disease.
In addition, consideration was given to the level of variation among control animals for

each factor being measured based on our own experience and reports in the literature.

Special attention was paid to variables we control, such as sex, age, and background strain. The statistical approaches to be used at the end of the study were an integral part of the experimental design process to ensure the robustness of all experimental approaches.

The experimental design was also reviewed and discussed with other researchers with experience in similar studies to further validate the design and ensure adequate control groups were included in each experiment, thus ensuring the validity of all data obtained.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

To maximize the data obtained from each animal, as many experiments as possible will be conducted longitudinally. For tissue harvest, all organs of interest will be harvested from each animal. Where possible, experiments will be designed to allow multiple follow-ups in the tissue from each animal, thus minimizing the total number of animals required.

Where relevant, and in most cases of therapeutic testing, small-scale pilot studies will be conducted prior to each full-scale study, with follow-up analyses to ensure that the treatment appears to be working as predicted and without any unexpected adverse effects.

This will also allow the subsequent experimental design to be optimized to minimize the use of animals. This approach will help to ensure that unnecessary large-scale experiments do not take place.

In addition, breeding strategies will be designed to ensure that as far as possible all animals from mating are used for an experiment. To maintain a line, animal breeding will be closely monitored and controlled to ensure the availability of sufficient mice while minimizing the birth of unnecessary ones.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are chosen for this project due to their genetic and physiological similarities to humans, which make them ideal models for studying neurodegenerative diseases such as motor neurone disease (MND) and Frontotemporal dementia (FTD).

Genetically modified mice are used to replicate key features of these diseases, including progressive memory or movement problems, providing a robust platform for understanding

disease mechanisms and testing therapeutic interventions.

These models are carefully selected and designed to ensure minimal pain, suffering, or distress to the animals. All mice are healthy at birth and only develop specific symptoms as they age, which mirrors human disease progression. While some animals may experience mobility issues, these are gradual and do not result in pain.

Additionally, experiments are planned to avoid significant long-term discomfort by prioritizing disease models with mild mobility issues whenever possible. In cases where more severe mobility problems are necessary for obtaining disease-relevant data, the animals' well- being is carefully monitored. Measures such as weight tracking, enhanced welfare checks, and providing a floor-level gel diet are implemented to minimize distress. Previous studies have shown that these care interventions significantly improve the quality of life for affected animals.

Minimally invasive methods are used wherever possible, such as brain or spinal cord injections, to ensure therapies reach their target sites. These procedures are well-tolerated, with animals typically recovering completely within a week. To further minimise harm, the project explores alternative delivery methods, such as non-invasive systemic AAV injections, which have the potential to eliminate the need for invasive procedures in future experiments and clinical applications.

To reduce the use of live animals, healthy control mice are only used when necessary. Where feasible, brain and spinal cord cells are sourced from previously euthanised animals, ensuring that no animals experience unnecessary distress as a result of the research. This approach adheres to the 3Rs principle (Replacement, Reduction, and Refinement), ensuring that the study is both scientifically robust and ethically responsible.

Why can't you use animals that are less sentient?

This project is designed to evaluate the effectiveness of new therapies for treating degenerative diseases such as motor neurone disease and dementia. These diseases affect the brain and spinal cord as we age and result in impairments in memory or mobility.

In order to test whether new therapies might be effective in human patients, we need to be able to show that they have an impact on the progression of the disease. To do this, we will use animal models that allow us to measure these behaviours as the animal age, so we can see if the disease mice receiving the treatment show improvement compared to those that do not. In addition, we will use multiple methods to measure the disease progression, such as behavioural tests and histological analysis, which will give us a comprehensive view of the disease. ALS and FTD onset occurs in aged people in the brain and spinal cord. Therefore, neonates or juveniles do not fit into this study. Moreover, the zebrafish or drosophila model is not able to be used for adeno-associated virus (AAV) injection as their brain structures are not similar to those of mammals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All animals in this project will undergo thorough monitoring and care to ensure their well-being. In addition to providing pain relief and local anaesthesia for brain injections, animals will be closely monitored for any signs of pain or discomfort for at least one week after

surgery (until recovered). Disease model animals that develop movement problems will be given special care, such as moistened food at floor level and regular weighing to ensure they have access to food and water and are healthy.

To minimize anxiety and stress, all animals will receive appropriate training for behavioural tasks before the start of experiments. This will also improve the consistency of data and allow for the use of the minimum number of tests and animals. Regular checks of NC3Rs website and liaison with NACWO and local NVS will be done for updates and implementation of changes as necessary.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

To ensure that animal experiments are conducted in the most humane and refined way, researchers engaged in this project will follow best practice guidance such as the ARRIVE (Animal Research Reporting of in Vivo Experiment) guidelines, 3Rs principles (Replacement, Reduction, and Refinement) and guidelines provided by organizations like the National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs) and the International Council for Laboratory Animal Science (ICLAS).

These guidelines provide information, training, and resources to support researchers in implementing the 3Rs, complying with regulations, and minimizing animal harm or distress.

Additionally, we will also engage with local legislation or regulations and ensure compliance with them.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will closely monitor the NC3Rs website for any updates and stay in regular communication with our NTCO (Named Training and Competence Officer) and others within the BSU who drive improvements in 3Rs approaches. By doing so, I will ensure that any relevant changes in guidelines or best practices are immediately implemented for all new experiments, and also consider making changes to ongoing experiments, as long as it does not compromise the integrity of the research outcomes. I will also ensure that any changes are communicated to all members of the lab, to ensure compliance and consistency in animal welfare practices.

Furthermore, I will use the 3Rs (Replacement, Reduction, and Refinement) principle as the basis for the experimental design, to minimize the use of animals while maintaining the scientific validity of the study.

64. Evaluation of an innovative vaccine platform for cattle

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Key words

Bovine respiratory syncytial virus, Vaccines, Cattle, Immunology

Animal types	Life stages
Mice	Adult
Cattle	Juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to provide proof-of-concept for a novel viral vector platform approach for cattle vaccines using respiratory syncytial virus (BRSV) in cattle as the initial candidate disease. This will involve evaluating the immunogenicity, safety and protective efficacy of novel viral vectored BRSV vaccine candidates.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Bovine respiratory syncytial virus (BRSV) is a major cause of respiratory disease in young

calves, which costs the UK calf industry ~£50M/year. More effective vaccines are required to better control BRSV and alleviate the significant animal welfare and economic burden the virus causes. Current commercially available BRSV vaccines include both live and killed, adjuvanted vaccines. A generic issue associated with these vaccines includes their inability to immunise effectively in the face of maternal and pre-existing immunity. In addition, there are safety concerns associated with both forms of the vaccines. In the case of live vaccines there is a risk of virus shedding and reversion to virulence. For killed vaccines the greater safety issues have been around local reactions and enhanced immunopathology following BRSV infection of vaccinated calves. More effective vaccines are required to better control BRSV and alleviate the significant animal welfare and economic burden they cause.

A novel viral vector platform, which is based on a naturally occurring, avirulent cattle virus, has been developed that can be adapted as a vaccine platform to contribute to the control of multiple infectious diseases affecting the cattle production industry. The virus vector can accommodate a large amount of genetic material, allowing several genes encoding foreign antigens to be included. The virus vector induces a strong immune response to the encoded foreign antigens, but only a limited immune response to the virus vector itself, allowing repeated usage without interfering with vaccine efficiency. The lack of interfering pre-existing vector immunity also allows it to be used in very young animals with maternal immunity. Furthermore, due to its replication mode, it can be administered mucosally or parenterally. Taken together, these attributes suggest the novel viral vector platform expressing BRSV antigens would be more effective than current commercially available BRSV vaccines.

What outputs do you think you will see at the end of this project?

Proof-of-concept that novel viral vectors expressing BRSV proteins can induce virus neutralising antibodies and protection against respiratory disease.

Providing vaccine candidates for further development as products.

All results from the project will be published in Open Access scientific journals once intellectual property has been protected.

Who or what will benefit from these outputs, and how?

In the short-term, the scientific community will benefit from knowledge on the performance of a new vaccine approach and its efficacy against BRSV in cattle. In the long term, this could lead to the development of a safer and more efficacious vaccine that results in enhanced BRSV control and consequently improved animal welfare and productivity in the cattle industry. BRSV infection often predisposes to bacterial superinfection, which requires treatment with antibiotics. A more effective BRSV vaccine strategy will reduce bacterial superinfection that requires antibiotic treatment, thus reducing antibiotic usage and antibiotic resistance. The long-term timescale for this is due to the fact that before therapeutic use the vaccine will have to licenced, which will require additional work (outside of this licence) to produce a registration application to the competent authority. Only then can the full benefits be realised.

In the medium-term, since the vaccine platform is easily adapted to target multiple infectious cattle diseases by insertion of a new pathogen antigen, the demonstration of its

efficacy against BRSV in cattle could lead to its use against other major cattle infectious diseases. Using a common and well- characterised vaccine platform reduces the cost of regulatory approval. This would bring benefits to policy makers involved in livestock disease control, the pharmaceutical and veterinary sector, and the general public through improved food security.

In the long-term, improving cattle production efficiency would reduce the impact of livestock-produced methane (a greenhouse gas (GHG)) as healthy cattle are more productive, with reduced emissions per head of cattle. A 10-percentage point decrease (e.g. 20% to 10%) in global livestock disease levels in a given year is associated with an 800 million ton decrease in livestock GHG emissions. This is equivalent to the average annual GHG footprint of 117 million Europeans (Animal Health and Sustainability: A Global Data Analysis). Reducing ill health in cattle through vaccination will increase productivity per unit and protect them from debilitating infectious diseases, thereby reducing overall methane emissions per unit of food produced.

How will you look to maximise the outputs of this work?

All outputs from this project will be published in Open Access scientific journals.

Outputs of this work will also be disseminated to end-users of cattle vaccines and influencers - producers, production companies, the animal health industry and specialist cattle veterinarians, through press releases, presentations at meetings/congresses and social media channels.

Species and numbers of animals expected to be used

- Cattle: 24
- Mice: 72

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The novel viral vector vaccine platform to be employed has been shown to be effective at inducing immune responses in rodents, rabbits, chickens, sheep, goats, and pigs. However, its immunogenicity and protective efficacy have not been evaluated in cattle.

The aim of the project is to evaluate the efficacy of the novel viral vector vaccine platform in cattle using a well-established experimental model of BRSV infection in calves. Calves are a natural host of BRSV infection, and the major protective antigen of BRSV has been defined. However, the most effective way of expressing the protective antigen from the virus vector needs to be determined. We will therefore use mice as a preliminary screen to assess the immunogenicity of the vaccine candidates as a guide to their selection for evaluation of their safety and protective efficacy in calves.

Typically, what will be done to an animal used in your project?

Mice used in this project will be immunised by injection of BRSV vaccine candidates subcutaneously or intraperitoneally. This will typically be conducted once or twice.

Samples of blood may be taken at intervals to monitor the immune response. The typical duration of such an experiment is 56 days. At the end of a study, animals will be culled humanely to assess immune responses in blood.

Typically, calves used in this project will be immunised by injection of a BRSV vaccine candidate into the muscle. As controls, calves will be inoculated with an irrelevant vaccine or saline. This will be conducted once or twice. Blood and nasal swab samples will be taken at intervals to characterise the immune response and to assess shedding of the vaccine virus. Vaccinated and unvaccinated animals will be challenged once by administration of BRSV by aerosol. Nasal swabs will again be taken daily for up to 7 days to quantify levels of challenge virus. Animals will then be culled humanely to assess lung pathology and tissues will be collected to assess BRSV loads and for further analysis of immune responses. The duration of an experiment is expected to be 50 days.

What are the expected impacts and/or adverse effects for the animals during your project?

No clinical signs of disease are expected for mice immunised with vaccine candidates. The impact vaccination and of blood sampling will be both mild and transient.

No adverse effects are expected following immunisation with BRSV vaccine candidates.

Mild to moderate clinical signs of disease for a few days' duration may be observed following inoculation of control calves with BRSV. This will most commonly present as a nasal discharge, coughing, a raised respiratory rate. An elevated temperature may also be observed. Clinical signs of disease are more likely to be observed in control calves than in vaccinated calves, if the vaccine is protective.

All animals will be clinically monitored both post-vaccination and -challenge. Assessments and interventions as appropriate will be performed at predefined frequencies in the experimental protocol, including euthanasia on welfare grounds if required. The impact of blood sampling, swabbing and inoculation of vaccine or virus will be both mild and transient.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The maximum expected severity for mice that are immunised with vaccine candidates is mild.

The maximum expected severity for unvaccinated control calves challenged with BRSV is moderate. The maximum expected severity for calves that are vaccinated and challenged with BRSV is mild.
It is estimated that 50% of calves will be in the mild severity category and 50% in the

moderate severity category.

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Due to the complex nature of the immune system, it is not currently possible to study immune responses to vaccination and to determine whether they are protected against infection without the use of animals.

Which non-animal alternatives did you consider for use in this project?

Cell culture-based systems will be used to generate the vaccine candidates and to evaluate induced virus-neutralising antibody levels and levels of virus replication.

BRSV glycoprotein expression by the recombinant viral vaccine vector will be confirmed in cell culture prior to the evaluation of the novel vaccines in animals.

Why were they not suitable?

No replacement options are available to replace the whole animal at this time as an entire organism, including the immune system, needs to be present.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Animal numbers to be used have been estimated using data previously collected from similar studies in consultation with a statistician.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Experiments will be designed to keep the animal usage to the minimum, but without compromising the validity of the research findings. The institute has a biostatistician and a policy that they must be consulted on the experimental design involving animals. For this project, information provided to them for determining groups sizes has included data

collected from other related mouse and calf studies as well as from peer-reviewed published papers (e.g. Sacco et al., 2022 Sci. Rep.12:22552 doi: 10.1038/s41598-022-26938-w; Valarcher et al., 2021, Vaccines 9:236, doi: 10.3390/vaccines9030236). To reduce potential future use, samples will be stored in a biobank, and we will maximise collection of samples post-mortem to facilitate further investigations without the requirement for additional animal experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In vitro models will be used to characterise novel BRSV vaccine candidates. Study design will be based on recently conducted relevant studies, which have been published in peer-reviewed journals (e.g. Sacco et al., 2022 Sci. Rep.12:22552 doi: 10.1038/s41598-022-26938-w; Valarcher et al., 2021, Vaccines 9:236, doi: 10.3390/vaccines9030236)). An inbred strain of mice will be used to reduce the genetic variability of the immune response induced by vaccination. Calves from a single farm will be used to reduce the risk of cross infection due to mixing of animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are being used to identify which form of the BRSV antigen produced by the novel viral vaccine vector induces the highest levels of neutralizing antibodies in order to guide the selection of vaccine candidates to evaluate in calves. Thereby reducing the numbers of calves undergoing unnecessary harm.

The use of adult inbred, SPF, mice as models for immunogenicity studies is well established, such that experiments can be designed with predictable outcomes that minimize pain, suffering, or distress, and avoiding confounding factors due to pathogens.

Mice will be inoculated with vaccine in the smallest volume commensurate with the aims of the procedure.

Sample collection will be carried out within clearly defined limits specified in individual experimental protocols, and repeated sampling will be done at frequencies such that the method of sampling causes no more than momentary pain and suffering and no lasting harm.

Mice are provided with red polycarbonate huts which provides them with a sense of security and shelter by reducing the level of perceived light, running wheels, tubes, fruit and vegetables, nest boxes and nesting material.

Calves will be inoculated with vaccine in the smallest volume commensurate with the aims of the procedure.

Calf experiments are designed with close consideration of the likely overall severity and the period of peak severity caused by administration of a defined and consistent dose of BRSV.

Following BRSV infection, calves may develop moderate signs of respiratory disease which will not exceed the specified humane endpoints. Scoring sheets specifically designed for signs of BRSV clinical disease in calves will be used. Symptomatic treatment as agreed with the veterinary surgeon will be provided to alleviate suffering whenever possible.

We have developed a purpose-built mask for aerosol administration which allows maximum exposure to virus in the minimum time.

Sample collection will be carried out within clearly defined limits specified in individual experimental protocols, and repeated sampling will be done at frequencies such that the method of sampling causes no more than momentary pain and suffering and no lasting harm.

Non-weaned calves will be housed in small groups in stables with bedding, access to hay and will be reared with a milk replacer.

Pre-study meetings involving the NVS, NACWO and animal services staff will be held to discuss any advances in animal care. Highly trained animal technicians will monitor animals throughout the day, ensuring they are comfortable and to maximise their welfare status.

Why can't you use animals that are less sentient?

It is only possible to confirm the best vaccine candidate for induction of an immune response by using an animal with a competent immune system. The use of adult mice as models for immunogenicity studies is well established, such that experiments can be designed with predictable outcomes that minimize pain, suffering, or distress. Mice will therefore be used as a preliminary screen to assess the immunogenicity of the novel vaccine candidates and guide their selection for evaluation of their safety and protective efficacy in calves.

Calves are the natural host of BRSV infection and provide the only model to evaluate the safety and efficacy of the selected candidate vaccine in protection against disease.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be acclimatized to their accommodation for at least 7 days prior to the start of any experiment and during this time will be familiarised to handling.

Animals will be housed together with bedding and other items of enrichment. Calves are housed in small groups to allow for normal social interaction. Highly trained animal

technicians will monitor the animals throughout the day, ensuring they are comfortable and to maximise their welfare. There will be increased monitoring for calves that develop clinical signs of disease. Symptomatic treatment as agreed with the Named Veterinary Surgeon (NVS) will be provided to alleviate suffering.

Pre-study meetings involving the NVS, Named Animal Care & Welfare Officer (NACWO) and animal husbandry staff will be held to discuss any advances in animal care. All experiments will be followed by a wash-up meeting to discuss all aspects of the study.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Stimulating the humoral responses of mice and cattle using antigens, and subsequent examination of the immune response are activities which have been extensively studied at our establishment.

Furthermore, the experimental calf model of BRSV infection is well established and has been used in studies to evaluate novel vaccine candidates and therapeutic compounds.

The procedures undertaken in this project will include administration of substances, withdrawal of blood, and nasal swabbing. The LASA published guidance, and other referenced best practice guidance will be adhered to including "A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes." Adherence to the ARRIVE guidelines for reporting these studies, as well as reference to the FELASA guidelines for calf health monitoring to help ensure the most robust health assurance for animals used in this study.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I regularly review information from the NC3Rs web site, the CAAT (Centre for Alternatives to Animal Testing), NORECOPA and newsletters as well as advances in procedures resulting in 3Rs such as from colleagues in the organisation or from relevant external publications which are regularly disseminated across the organisation via an internal newsletter. As an experienced laboratory we will be in regular contact with other researchers world-wide and new knowledge on procedures of similar studies will be taken into account. Relevant advances will be incorporated into study designs and procedures.

65. Harnessing systemic and local immunity against infections and cancer

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Liver cancer, Hepatitis B, Sars-CoV-2, Immunotherapy, Vaccine

Animal types	Life stages
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to investigate the essential players involved in immunity to Hepatitis B Virus (HBV), liver cancer and SARS-CoV-2 infection and to explore strategies to boost immune responses towards protection or control of these diseases. In particular, we aim to further our understanding on the contribution of immune cells residing within tissues and the interplay of different cell types in these disease settings.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This project investigates diseases that are major global health problems. Chronic hepatitis

B and liver cancer have high incidence and mortality rates, respectively, pointing to the failure of current therapies that require life-long treatment, present toxicity and are not completely curative. SARS-CoV-2 remains a global health priority and current vaccines are limited in completely blocking infection, particularly against different variants of the virus. This work will enable the development of more effective vaccines and therapeutic interventions for HBV, liver cancer and SARS-CoV-2 by extending the knowledge on immune responses and immune regulation in these diseases and allowing testing of novel approaches. Also, this work will increase the understanding of liver and local immune suppression in chronic hepatitis B (CHB) and liver cancer that may be applicable to other diseases and cancers with potential to improve diagnostics and inform the choice of therapeutic approaches.

What outputs do you think you will see at the end of this project?

This project will provide new insights into the influence of the liver and tumour microenvironment in chronic diseases and the role of circulating vs local immune responses in tissues such as the liver, lungs and tumours. We believe these findings can inform patient treatment and diagnosis in liver cancer and the aid the development of novel vaccines or immunotherapies to liver cancer, chronic HBV and SARS-CoV-2. Particularly regarding COVID-19, we expect to extend our data on the development of a next generation SARS-CoV-2 vaccine that would be suitable for clinical testing in around 3-4 years from the beginning of this study. At the end of this project we expect to achieve publications in high- impact journals to communicate the novel findings expected for the research topics listed below:

Mechanism, predictive markers and discovery of molecules that can be targeted in CHB and liver cancer
Our approaches addressing the mechanisms of hepatic immune suppression will allow us to define predictive markers and identify molecules that can be targeted by immunotherapy, contributing to improved diagnosis and treatment of patients with CHB and liver cancer. Increasing the understanding of how to overcome immunosuppression in HBV and liver cancer can contribute to improve outcomes in other infections of the liver (HCV, malaria). Conversely, it can inform how to silence immune reactivity in autoimmune liver disease and liver transplantation. We will compare regulation of T cell responses in the liver to those in the lung to identify unique and common mechanisms of immune regulation. Our findings will therefore contribute to the fundamental understanding of therapeutic strategies in all these fields.

Developing novel immunotherapeutic strategies
Our work will allow us to develop new immunotherapeutic approaches or combinations of treatments to boost immunity against CHB and liver cancer inducing immune responses that can target infected or tumour cells specifically while modulating liver and tumour-mediated suppression. Our work will focus on T cells, B cells and NK cells, which are vital populations in combating infections and cancers, particularly those that are resident in the liver and tumours. We will test immunotherapies or therapeutic vaccines that are either already in clinical testing (and can be repurposed) or that have a fast developmental pathway to accelerate clinical translation.

New generation SARS-CoV-2 vaccines
We expect to have developed a new generation SARS-CoV-2 vaccine not only based on Spike as current formulations, but including more conserved parts of the virus and,

therefore, allowing for protection against different variants or other animal coronaviruses. Also, we will have assessed the protective benefits of this vaccine being delivered by the mucosal route to induce local immunity in the airways. We expect this to be essential to elicit more efficient, rapid and durable viral control in the lungs. Findings may be applicable to the induction of mucosal immunity against other respiratory pathogens beyond the coronavirus family.

Who or what will benefit from these outputs, and how?

We expect outputs from this project to be disseminated as publications and presentation in conferences and meetings within the research and clinical communities. In a shorter to medium term, this will extend the knowledge on hepatitis B, liver cancer and SARS-CoV-2, contributing to future work of other research groups in these fields and the pharmaceutical industry. Also, dissemination of our work in impactful journals and conferences will contribute to strengthen the productive track records of our group and help the career progression of students and researchers. In a longer term, we expect this project to directly impact patient care by supporting clinicians with best indicators of treatment predictions and ultimately by generating novel vaccines or immunotherapies.

How will you look to maximise the outputs of this work?

We will present our findings at relevant national and international conferences and strive for publications in high impact journals (we will prioritise open-access publications). We will also aim to share negative results in publications and conferences as much as possible when they provide useful information to our field.

We will maintain our close relationship with the clinical teams relevant to our research aims to facilitate the translational aspect of our work. We will look into possibilities of filing patents for new technologies and seek collaborations with companies to accelerate drug development. We will also continue our extensive patient and public involvement (PPI) work to ensure involvement of relevant communities and stakeholders.

Species and numbers of animals expected to be used

- Mice: 3200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The main purpose of our study is to find novel strategies to stimulate the immune system to fight HBV, liver cancer and prevent infection with future SARS-CoV-2 variants and novel coronavirus zoonoses, with a particular focus on harnessing local immunity in the organs that these diseases affect (liver and lung). For this, we need to test therapeutic and interventions and investigate mechanisms of immune suppression in HBV and liver cancer, which require studying immune cells in the context of their complex interactions with other cells and molecules present in the tissue microenvironment.

Animal models are essential for these studies, since analyses of immune responses in vitro limits our understanding of the interplay of the components of the immune system and the testing of new therapeutic strategies. Murine models that recapitulate some key features of HBV or liver cancer have been developed and are established in our laboratory. Adult mice are also appropriate to test immune responses to vaccines and the possibility of working with genetically altered animals allows for a deeper understanding of key molecules or cells or increase the translational relevance of the work, for instance by predicting T cell responses in patients using mice that express human MHC. We will use the proposed animal models to investigate the relationships between different immune components in vivo and in vitro by studying the liver, lungs and other tissues directly. For example, this approach will allow us to assess the dominant in vivo effect of NK cells (for which we have identified several potential roles from our human in vitro studies) and their influence on T cell responses. The mouse model also allows more extensive functional analysis of specialised tissue-resident cells, informing how to harness or modulate these in with novel immunotherapeutic targets. Importantly, the animal model will allow us to test and refine therapeutic interventions and new generation vaccines against SARS-CoV-2.

Vaccines and new therapeutic strategies will only be tested in animals once we have an indication of their relevance from our human work and if they are suitable for clinical translation. The main scientific questions we plan to explore using these animal models were first raised from studies performed by our group and others using patient samples (and in vitro culture models where possible), allowing us to focus our animal studies on the mechanisms likely to be most relevant to disease outcome in patients.

Typically, what will be done to an animal used in your project?

Our project proposes to study immune responses in hepatis B virus infection and liver cancer, aiming to further the understanding of the interplay of different immune cells and expanding the rationale on the development of new immunotherapeutic approaches. We plan to investigate these using mouse infection models of HBV that recapitulate different characteristics and disease stages observed in patients. We also plan to study the induction of immune responses in the context of liver cancer, using HCC mouse models based on the implantation of human or mouse tumour cells subcutaneously or intrahepatically. We will also work on the development of a next generation SARS-CoV-2 vaccine that we expect to induce more broad and durable protection than existing vaccines and to be delivered by mucosal route to induce local immunity in the airways.

For these studies we have delineated four protocols that will typically involve:
Breeding and maintaining genetically altered mice to be used in the other protocols.

Animals will be bred by mating in pairs (or occasionally trios) on a number of occasions. Offspring will be maintained by methods appropriate to their genetic alteration until they reach a maximum of 15 months of age. Animals are not expected to show harmful phenotypes.

Most animals in this protocol will receive the HBV infection model and later be treated with therapeutic vaccines and/or other immunotherapeutic interventions (typically 2-3 doses). Blood samples will be collected to confirm establishment of chronic infection. At the end of the experiment (generally 30-40 days after infection) mice will be euthanised and organs will be harvested for downstream analysis.

Mice may be subjected to tumour cell implantation followed by treatment at different time points (typically 2-3 doses). We estimate that 70% of animals used for our liver tumour research would be directed to studies employing subcutaneous tumours and 30% to orthotopic liver tumours that, currently, require surgical techniques. Tumour growth will be monitored, and blood samples may be collected (in an estimated 40% of animals). At the end of the experiment (30-40 days after tumour cell injection) mice will be euthanised and organs will be harvested for analysis.

Naive mice will be immunised with 1-3 doses of candidate prophylactic pan-coronavirus vaccines by the intramuscular or mucosal route. Blood samples will be collected after each dose. At the end of the experiment (typically 14 days after the last immunisation, but in an estimated 20% of animals 60-90 days after the last dose) mice will be euthanised and organs will be harvested for analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Genetically altered animals are not expected to show harmful phenotypes. Some animals may have an altered immune system making them more susceptible to infection and, therefore, will be housed in a barrier environment, thereby minimising the likelihood of compromising health.

The models of HBV infection we utilise do not induce clinical signs in mice. Similarly, immune interventions to be tested have been previously shown to be safe and well tolerated in mice and/or humans.

Injected viral vectors and immune interventions are expected to be non-toxic or have limited toxicity at the doses used. Injections are not expected to cause more than transient pain or discomfort.
The growth of tumours can cause distress or pain due to local effects, so tumour burden will always be limited to the minimum required for a valid scientific outcome and by defined humane endpoints. For subcutaneous tumours, mean diameter should not normally exceed 1.2 cm which typically occurs 30 days after tumour cell injection. Mice will be monitored daily for signs of toxicity or behaviour changes and weight will be assessed 2-3 times a week following injection. If clinical signs indicate potential adverse effects, animal will be humanely culled and vigilance increased for the remainder of the cohort.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We expect 25% of the animals to experience mild severity and 75% moderate.

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our work is highly translational and includes testing new therapeutic and prophylactic interventions against extremely prevalent life-threatening diseases.

Animal models are crucial to test efficacy of new therapies in vivo, including allowing assessment of the optimal route of administration and dosing schedule. The development of immunotherapies for cancer and chronic infections also require the investigation of mechanisms of suppression triggered during chronic HBV and liver cancer to find novel and relevant pathways that can be targeted. These studies involve a complex network of cell-to-cell interactions or molecules present in the tissue microenvironment that can only be fully replicated in animal models currently.

Similarly, our lung mucosa targeted pan-coronavirus vaccine needs to be optimised through in vivo murine studies to overcome local tissue tolerizing mechanisms. In vitro studies using human or murine tissue will be used whenever possible. New therapeutic strategies will be tested in animals only if we have an indication of their usefulness gained from our human work.

Which non-animal alternatives did you consider for use in this project?

Patient studies form the bulk of our laboratory's research and will inform all of our animal work. Whenever possible we will make use of human tissue samples in order to reduce the number of animals for our study. We have a well-established collaboration with clinical teams from the Royal Free Hospital in the areas relevant to our research. Alongside this, we have developed different tools to study immune responses in vitro using human samples. To overcome the difficulty of studying antigen- specific T cells in chronically infected patients and cancer, we developed engineered human T cells and NK cells that will be used to test the efficacy of different immunotherapeutic strategies in vitro.

Additionally, we have recently established an in vitro infection model using human Hepatoma cells expressing the HBV entry receptor NTCP in our laboratory. We are also working with a spheroid model to study anti-tumour responses using a 3D model. We have developed an in vitro immunisation assay using PBMCs and aim to adapt this to cells from human lymph nodes. Whenever possible, immunotherapeutic candidates will be tested using in vitro systems and engineered human T and NK cells and only the most promising approaches will be tested in vivo.

Why were they not suitable?

Although we have a reliable source of human samples, access to relevant diseased tissue, such as the HBV-infected liver or liver cancer is limited. Attempts to recreate 3D culture systems mimicking the complexity of liver tissue or tumour interactions have not been successful thus far. In vitro vaccination studies do not allow for investigation of the efficiency of antigen presenting cells in different tissues or evaluation of immunisation

routes to induce tissue-specific immunity more efficiently.

To date, there are no in vitro models that allow us to selectively deplete or replace immune subsets and pathways in a relevant disease context to allow accurate therapeutic target evaluation and safe pre-clinical testing. In summary, the study of immune responses in vitro greatly limits our understanding of the interplay among the components of the immune system and of new therapeutic strategies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Careful consideration has been given to the minimum number of mice needed to obtain statistically significant results. The number of animals needed was estimated to reflect the animal work we have proposed in our current and most recently obtained research grants, projecting that we would need similar numbers going forward until completion of the 5-year period of duration of this license. Our estimates are also based on our previous work, taking into consideration that we have now expanded our initial murine research on CHB into hepatocellular carcinoma (HCC) and SARS-CoV-2. This also means that animal work under this license will reflect the research projects of at least 4 postdocs and 4 PhD students.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have considered the variability inherent to each model and considered group sizes that would be sufficient to find differences in outcome. For designing each protocol within our research projects, we have used power calculations to estimate mouse numbers.

To minimise experimental bias, test subject blinding and randomization will be applied as much as possible. Measures will include using both mouse genders and animals of similar weight and age from the same source, distributing experimental groups across multiple cages, minimising experimenter or handler turnover. Data will be confirmed using rigorous statistical analysis.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Each animal will be analysed in as many ways as possible, without increasing animal suffering, to reduce the numbers needed for experiments (e.g. using repeated monitoring, non-invasive imaging).

Since the aims of our projects are often linked, we will utilise the same animals whenever possible, e.g. to study multiple immune cells purified from the same mouse or using

different organs. Uninfected or infected untreated animals might also serve as controls for two different scientific questions.

We will also keep up to date with current developments in our field in order to refine techniques, reduce animal usage and avoid unnecessary duplication of experiments.

By using multiparameter flow cytometry (staining for 16-30 different immune markers in each sample) we will maximise the information gained from each sample. We can also indirectly measure liver damage and HBV activity using serum ALT and HBV serology. Taken together these measures will allow us to monitor mice using venesection on several occasions before needing to cull the respective mice to harvest their liver, thereby substantially reducing the overall number of mice used.

We will also seek to perform bulk and single-cell RNA sequencing and TCR/BCR sequencing to maximise the data extracted from each sample.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The only species of animal that can be naturally infected with human HBV are chimpanzees. Woodchucks can be infected with a hepatitis virus similar to human HBV and have provided some insights. However, their use is limited due to their outbred nature, hibernation pattern, their relatively big size and their immune markers being ill-defined. Mice, on the other hand, have well-defined immune markers but they are not natural hosts for HBV. A novel route of packaging HBV into an unrelated viral vector that targets the murine liver following injection of a well-tolerated volume of viral inoculum can now achieve chronic infection of mice. This model can be adjusted to either achieve an acute or persistent infection, allowing dissection of the mechanisms governing the two different outcomes. We have therefore chosen this model, as it will be the most suitable for our translational HBV work. This infection model reproduces tolerogenic or suppressive pathways observed in patients but does not induce expected clinical features.

The use of C57BL/6 mice that are transgenic for the human HLA-A2.1 and HLA-DR1 and deficient for the murine H-2 class I / class II will allow us to track antigen presentation and CD8 and CD4 T cell responses respectively using highly specific tools developed for well-defined HLA-A2 and HLA-DR1- restricted HBV, HCC and SARS-CoV-2 epitopes in humans (in parallel with our studies in HLA- matched patients).

Neither acute nor chronic HBV infection have been reported to lead to any severe suffering in these murine models. The HLA-A2 transgenic mouse model and others that we propose to use have not been found to manifest any ill-effects.

During the course of our current licence using the AdHBV infection model, mice did not show signs of pain or suffering. Also, the therapeutic interventions and vaccines tested to be tested in the new license will be based on similar compounds that did not show signs of toxicity during the current license. The immunomodulatory molecules to be tested have been tested in clinical trials for different diseases or have been published to be safe.

The growth of orthotopic tumours is more likely to cause pain or weight loss than subcutaneous tumours. Also, tumour cell injection into the liver requires a small surgical procedure. Therefore, we will prioritise testing therapeutic interventions using the subcutaneous model and only test the most promising therapies in the orthotopic model.

We are able to follow tumour growth by in vivo non- invasive imaging and will use this to avoid excessive tumour burden.

In the event of unexpected adverse effects, we will use humane endpoints as outlined in our protocols.

The handling and procedures will be done by an experienced researcher to minimize distress. Animals will be kept in well-maintained housing, with environmental enrichment such as nesting material provided, which will reduce experimental variability caused by environmental stresses such as infections.

Why can't you use animals that are less sentient?

Adult mice are the smallest animals with most similar liver and lung physiology, structure and immunology to humans and therefore represent the best life stage to perform our studies. In vitro studies will be used to address our research questions initially and will inform the animal work, serving not only to reduce animal numbers, but also to define which pathways or therapeutic interventions have higher translatable potential.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have been performing similar experiments for a number of years and have optimised procedures to minimises distress and pain to the animals. For instance, we have started following recommendations to perform intramuscular injections under inhaled anaesthesia and, similarly, tumour cell transplantation by subcutaneous route preceded by shaving the hair in the area to facilitate tumour monitoring. Additionally, we have worked with our NACWO and NVS to carefully optimise our anaesthetic and analgesic protocols for pre- and post-operative care and will continue to seek their help to improve our techniques.

At all stages, we have found good tolerance of the surgery itself, however, we are seeking funding sources and organising our facility users to acquire an ultrasound system that would allow for injection into the liver without need for surgical procedures. Such equipment would also allow for more precise tumour measurements and follow up at different time points with non-invasive techniques.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Regarding best practice for laboratory animal anaesthesia, analgesia, intraoperative care

and post- anaesthesia support we will follow the NC3Rs guidelines. Additionally, our work on cancer models is in compliance with the best practice defined in the “Guidelines for the welfare and use of animals in cancer research” (Workman et al., British Journal of Cancer (2010) 102, 1555-1577). We will be advised by our NACWO and NVS regarding best practice guidance for animal welfare and environmental enrichment and will work closely with the animal facility staff to make sure recommendations are followed and updated when necessary. With the support from the BSU staff we will continue to carefully and routinely monitor our animals for adverse effects.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will continue to be updated by our NC3Rs newsletter and our institutional 3Rs newsfeed. Also, our close interactions with our NVS, NACWO and the staff at the Biological Services unit will allow us to learn about additional advances that are communicated to them and will help us to implement changes. We will also communicate advances that can be relevant for other laboratories to other groups in our department using a platform that we have created for groups performing animal work.

66. Mitigating against life-course complications of heat stress

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Heat, Embryo, Development, Porcine, Epigenetics

Animal types	Life stages
Pigs	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To establish the nature, extent and persistence of developmental abnormalities in mammalian embryos (and offspring) following transient heat exposure during the period around and shortly after conception (termed the periconceptional period). Persistence in this context relates to intergenerational (F1 to F2) transmission of epigenetic and cellular irregularities that may lead to physiological abnormalities in offspring (F1); this will also require a cohort of F0 pregnancies to go to term and a proportion of F1 progeny reared to sexual maturity and bred to generate F2 embryos

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Epidemiological evidence in humans indicates that acute adverse heat exposure during the pre- and peri-implantation period results in pregnancy loss, with potential long-term consequences for offspring health and wellbeing, although these are poorly understood beyond the neonatal period. Similar effects of acute heat stress on embryo survival and fertility are seen in production animal species such as cattle and pigs. Identifying underpinning mechanisms requires appropriate animal models. Farm animal and human embryos share many aspects of early development including gene regulatory networks, developmental timing, and signaling requirements for cell-lineage establishment. Thus, in addition to being species of clinical importance in their own right, they represent excellent models of human embryogenesis.

What outputs do you think you will see at the end of this project?

Other researchers in the UK: the results and data will be published open-access in peer-reviewed clinical journals. Therefore, the information will be disseminated amongst scientists and clinicians with an interest in the longer-term effects of heat-stress. The short-term impact will be mainly academic via discovery of fundamental mechanisms of how heat exposure potentially alters health in the next generation and how to mitigate these effects. In the medium-term, this project will impact on health strategies in at-risk populations. In the longer-term, the project has the potential to impact future generations by ensuring babies born during a period of heat-stress have the best chance to go on to live healthy lives.

International researchers: This project will create opportunities to expand the climate challenge research community internationally, i.e. to those countries most at-risk for experiencing acute heat exposure. The biobank generated as a result of this project will provide a resource for other researchers to test their novel molecules or pathways.

Technology: The project will generate novel in-vitro 3D models to test heat exposure effects on endometrial function - established using cell lines. Custom made in vitro microfluidic platforms combining extracellular scaffolds, microfluidics, and thermal control systems that effectively generate an 'endometrium-on-a-chip' will be made available to other researchers after publication.

Who or what will benefit from these outputs, and how?

The short-term impact of this project will be mainly academic via discovery of fundamental mechanisms of how heat exposure potentially alters health in the next generation and how to mitigate these effects.

In the medium-term, this project will impact on health strategies in at-risk populations.

In the longer-term, the project has the potential to impact future generations by ensuring babies born during a period of heat-stress have the best chance to go on to live healthy lives.

How will you look to maximise the outputs of this work?

Results will be disseminated to academic colleagues at key International conferences and

open access publications/repositories. A data management plan has been produced detailing these impacts. Outreach to affected communities will be via links with clinical colleagues (e.g. Royal College of Obstetrics and Gynaecology).

Dissemination to affected communities will be via the project teams links with the United Nations. Outreach to the general community will be facilitated via press releases, and engagement in established knowledge exchange activities e.g. Pint of Science.

Species and numbers of animals expected to be used

- Pigs: 125

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Epidemiological evidence indicates that acute extreme heat exposure during the peri-implantation period results in pregnancy loss in humans. Even for successful pregnancies there can be consequences for the long-term health of the next generation. One of the key organs that can mediate these effects is the uterine endometrium as it functions to: 1) support embryo development via transport of molecules, 2) responds to molecular cues from the embryo, and 3) provides biophysical interactions that support implantation. In order to identify underpinning molecular mechanisms of these interactions in the face of acute heat exposure requires a combination of approaches, some of which inevitably need to be tested in appropriate animal models. As a result of research from our larger project team, then the early development of pig and human embryos share many similar aspects of early development including gene regulatory networks, developmental timing, and signalling requirements for cell-lineage establishment. Thus, pigs represent an excellent model of human embryogenesis, but also of human physiology (cardiovascular, renal and metabolic systems are very similar). The periconceptional period and environment can epigenetically alter embryo development with long lasting effects on offspring. Moreover, the pig is a precocial litter-bearing species allowing sexually dimorphic effects of heat exposure to be determined in offspring from within a litter, reducing variability and controlling for genetics.

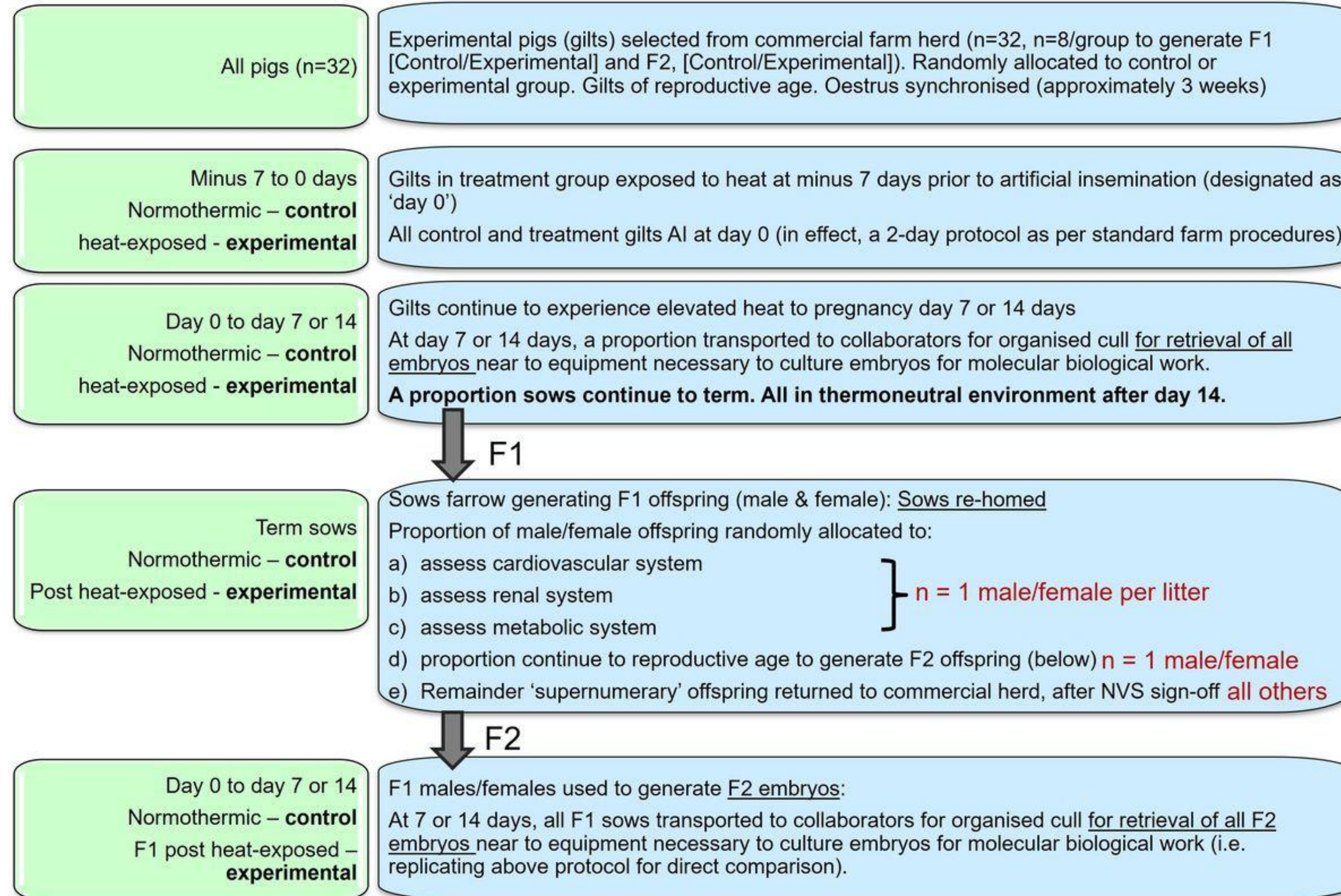
Typically, what will be done to an animal used in your project?

Protocol 1: Typically for any individual animal on this licence, then up to one month before heat exposure a gilt will start to receive hormones to synchronise oestrus using standard veterinary procedures as used for any commercial herd (may take 18-20 days). At minus 14 days, animals (treatment group only) will be moved to designated large rooms and acclimatized to heat exposure with the experiment beginning at minus 7 days. At minus 2 to 0 days (these days being point of artificial insemination, using semen from a single sire) heat-exposed gilts will continue to experience an elevated environmental temperature (controls will continue to experience normothermia in a separate designated space). Day 0 will essentially be designated as day 0 of pregnancy. After, to plus 7 or 14 days of pregnancy, the pregnant sows will continue to experience either normothermia (control

group) or an elevated environmental temperature (i.e. 'heat-exposed', as determined by temperature-humidity- index or THI). A proportion of sows will be S1 culled at these timepoints for retrieval of embryos, which will enter into in vitro protocols. All remaining sows will continue beyond day 14 pregnancy to term in normothermic conditions until littering. Sows will rear litters to weaning in designated spaces as per normal farm practice and in Home Office designated spaces. At weaning, sows will be NVS checked for return to the commercial herd. Weaned offspring (male/female) will remain on licence in designated spaces until adulthood (50-70kgs) where a proportion will either a) be transported to collaborators
for physiological measurements (as described in Protocol 2), b) remain on licence to be artificially inseminated for generation of F2 embryos (day 7 and day 14) or c) be returned to the commercial herd after NVS sign off.

Protocol 2: Typically for any individual animal on this licence, then a male and female F1 offspring at a young-adult age (6-7 months, 50-60kg body weight) will be sedated prior to general anaesthetic for insertion of vascular catheters. The animal will then be recovered to a pen and have their blood pressure recorded by telemetry 24/7, and on separate days have agents injected into their blood to allow measurement of renal and metabolic function. The animal will then be schedule 1 culled for retrieval of tissues.

Typical Lifecycle for animals on this licence.



What are the expected impacts and/or adverse effects for the animals during your project?

To minimise suffering: All experimental animals will be monitored for good general health, according to an adverse effects monitoring sheet. For protocol 1 no invasive procedures will be conducted, other than the administration of hormones for artificial insemination and retrieval of embryos will only be after schedule 1 cull. During the heat exposure period, animals will be monitored for signs of heat-stress, as appropriate for pigs and as designated on a monitoring sheet. Higher THI have been implemented in pigs in other studies without any significant adverse effects being noted. In order to monitor and control the climate experienced by pigs on licence during heat exposure, we will use a SKOV LPV (Low Power Ventilation) system paired with heat sources (e.g. overhead 1.5kw element heaters) to regulate heat for individuals in thermoneutral (18-20°C is thermoneutral for pigs, corresponding to a thermal heat index [THI] of ~65) or heat-exposed (~28-33°C with day/night fluctuation as appropriate, corresponding to a THI of approximately 85). These conditions are equivalent to the heat stress experienced by disadvantaged communities in developed countries during a heat-wave. The system manages the THI within 2-5% boundaries and monitors excess noxious gas build up within rooms
For protocol 2 invasive procedures will be conducted, but these are relatively simple and the animal will receive anaesthetics and analgesics to manage the discomfort they may experience. Long-acting antibiotics will also be administered to prevent catheter related infections and catheter failure.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

50% of sows will experience a thermoneutral environment (considered 'mild') with the remaining 50% experiencing a brief period of elevated ambient temperature (also considered mild). For offspring from each group of sows, then approximately 20% (1 male, 1 female from expected litter size of n=10) will experience moderate severity protocols, related to undergoing general anaesthesia for implantation of recording devices.

What will happen to animals used in this project?

- Rehomed
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The proposed work aims to investigate the impact of heat exposure during pregnancy on developing embryos (and ultimately offspring well-being). This will be achieved using pigs as a model species. Developmental events at a molecular level are similar between pigs and humans and it is important to examine these effects in utero, as well as alongside in vitro and in silico approaches. The longer term consequences of early heat exposure are important to measure in the whole animal as blood pressure, renal function and

metabolism are clearly influenced by multiple body systems.

Which non-animal alternatives did you consider for use in this project?

The wider project which involves other researchers and other institutions extensively use non-invasive technologies and non-animal alternatives to complement this study. The project takes an interdisciplinary approach by harnessing the groups expertise in 1) bioengineered embryo-maternal interaction models, 2) comparative developmental biology of the pig and mammal systems, 3) computational evolutionary biology, we will determine conserved mitigation pathways for intervention in humans to understand the mechanistic biology of heat stress and to identify potential intervention strategies.

Why were they not suitable?

The non-invasive technologies are available and suitable for studying isolated feto-maternal simulated interfaces and in silico analysis of the genomes of, for example, heat-adapted species. This project will also use cell culture systems to complement the in vivo work. Ultimately, the effect of heat exposure needs to be examined in the whole animal as for final pre-clinical discovery science or testing with a view to translation into clinical practice/ clinical relevance for at-risk populations, then studying in vivo in the live animal is required. The primary effects to be examined in this licence relate to a whole body physiological response (to heat exposure) and for this reason it is important to utilise the most appropriate animal model and then establish the causative factors in the (materno-fetal) circulation that may not be present in a culture dish, such as hormones or anti-sense RNA. With isolation of such putative causative agents then future in-vitro studies are much more realistic.

This is not done lightly and all other options are always considered. One strength of the current wider programme of work is that we will combine these technologies with techniques that are not able to be performed, ethically, on humans e.g. embryo recovery and development in culture. The degree of heat exposure of the animals is similar to that experienced by many production animals in countries around the world that experience acute episodes of adverse heat.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Objective 1: The sow is the biological unit of replication (i.e. the experimental unit to which heat exposure is applied). For our first objective, we require embryos at day 7 and 14. We estimate n=8 pregnant gilts per treatment per time-point (i.e., 32 pregnant gilts in total for this objective).

For Objective 2: The sow is the biological unit of replication (i.e. the experimental unit to

which heat exposure is applied). For our second objective, we require F1 offspring (n=1 male/female from each sow; n=8 sows per treatment group). In addition, a further n=4 pregnant gilts per treatment group will be required to enable production of F1 pregnant females (i.e., 24 pregnant gilts in total for this objective). Weight gain will be monitored in all offspring. A proportion will have physiological measurements conducted included body composition. Offspring not used in any procedure will be signed off the licence and returned to stock. A proportion of F1 males/females will be used to generate F2 offspring, as detailed in Objective 2, Protocol 2:
Objective 2, Protocol 2: Reciprocal mating schedule between F1 boars and gilts required to generate F2 Day 14 gastrulating embryos. The arrangement is required to avoid half-sib matings in our outbred model species. Thus, sexually mature F1 female offspring derived from a known breeding pair (from F0 control or treatment groups, semen from 'Sire A') will become pregnant using semen (artificial insemination) using semen from a different but known boar (e.g. 'Sire B') (as described in Figure 1, research plan). The output (i.e., 4F2(♂+♀)) refers to 4 'mating-pairs' and a single male and female embryo from the litter of each mating-pair. F2 mating-pairs compare Heat Exposed to Control treatment groups (in line with F1 comparisons). However, F2 mating-pairs will also represent Heat Exposed male/female F1 offspring mated to Control female/male F1 offspring. Thus, in addition to confirming and quantitatively describing the persistence of developmental and epigenetic defects in F2 embryos, this experiment will inform on the direction of parental inheritance of epigenetic and developmental outcomes (i.e., whether they are passed down the male or female line, or both lines). In this objective, we estimate requiring a further n=32 pregnant gilts.

This equates to a total of n=88 pregnant gilts. However, estimating approximately 75% reproductive success rate, we envisage requiring no more than 125 gilts over the course of this licence.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Aspects of study design and outcome are always taken into account and are pre-determined in discussion with animal bioscientists. Power calculations are performed as necessary, based on aforementioned known variabilities in outcome. The PPL holder helps many other scientists with their statistics and uses the EDA for teaching undergrads study design. The PPL holder holds 'Stats club' sessions on an as needed basis for staff and undergrads. As an example, factorial designs are used to achieve greater statistical power with lower numbers of animals per group. For quantitative data, I always conduct a power calculation to determine optimal sample size and keep animal numbers to a minimum whilst maintaining statistical significance (alpha set at 0.05) and study power (>80%).

For example, in this study, we have accounted for the most variable parameter when undertaking our power calculations and have powered the experiments accordingly.

Individual adult gilts are the experimental unit for F1 embryos (n=8 Control; n=8 heat exposed) after artificial insemination. Gilts will proceed to gestation day 7 or 14 whereupon embryos will be recovered for molecular biology and in vitro culture experiments.

Experimental variance will be kept to a minimum by first considering male and female fetuses (i.e siblings) as the outcome of interest which will have shared a uterine

environment. Two sires will usually be used to reduce the main source of variability in our primary outcomes. Gilts will be randomised to treatment groups using the randomise function in MS Excel. For each and every experiment, as part of good laboratory practice, we write an experimental protocol which includes: a statement of the objectives, a description of the experiment to be conducted including treatment groups, animal numbers and experimental material, an outline of the analysis and principal experimental outcomes.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will only purchase (from within the closed established breeding herd) and enter into any protocol the required number of animals as per the study design. For any potential new protocol, then the design will include step-testing of one animal or batch, followed by further batches once the new new protocol has become established. Any tissue required by others will be biobanked and shared as the PPL holder has done in the past with multiple groups. Supernumerary embryos will be cryopreserved and fixed for later incorporation into other experiments.

Supernumerary control or heat-exposed offspring will be checked by the NVS and will be returned to farm stock if deemed appropriate.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Identifying underpinning mechanisms requires appropriate animal models. Pig and human embryos share many aspects of early development including gene regulatory networks, developmental timing, and signalling requirements for cell-lineage establishment. Thus, pigs represent an excellent model of human embryogenesis and offspring development to adulthood. The periconceptional environment can epigenetically alter embryo development with long lasting effects on offspring. Moreover, the pig is a precocial litter-bearing species allowing sexually dimorphic effects of heat exposure to be determined between half-siblings.

However, mechanism of action studies are limited due to inadequate in vitro systems appropriately mimicking embryo-maternal interactions in vivo. Recent developments allow us to directly test in vitro how heat-exposure affects endometrial function and embryo development up to gastrulation.

Harnessing these in vitro data with computational evolutionary approaches will allow us to define what components of the heat stress response are unique or common, divergent or conserved between human, pig and other mammals. This interdisciplinary, comparative

approach will generate in vivo maps of heat exposure, complementing in vitro and in silico approaches to identify molecules/pathways that will guide mitigation strategies to protect the next generation.

Why can't you use animals that are less sentient?

In order to examine specific mechanisms for the effect of heat exposure on developmental mechanisms then an appropriate mammalian model is required. The early developmental events between pigs and humans have been shown to be more similar than between rodents and humans and thus for periconceptional events, then pigs are the appropriate animal model. The size of their organs (e.g. endometrium, uterus) in relation to body weight is near identical to human - this is an important consideration when studying physiology and embryo-endometrial interactions. The proposed work will be conducted alongside other non-animal approaches.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The PPL holder will always consider new, better methods that help reduce any animal suffering and improve study outcomes. For example, we altered our anaesthetic regime for pigs in consultation with the NACWO, named vet and veterinary consultants when it was apparent that a different regime produced better study outcomes (e.g. improved post-operative recovery). Equally, we also sought to reduce unnecessary harm to sham-controls (i.e. no mid-line laparotomy) when it was apparent they were similar to other controls that had undergone full sham-surgery.

Animals will either be housed in groups to help reduce stress or if that is not possible and they have to be housed individually, then they will always be in sight and sound of other pigs. Any signs of distress as a result of heat- exposure will be monitored daily using a state-of-the-art environmental monitoring system. Intervention points will be adopted e.g. intervention will occur when measurements are deemed outside normal parameters including panting and huddling scores and will be taken under the advice of the NVS. Intervention could, for example, take the form of removing individual gilts from heat exposure. If an individual does not respond to intervention, in consultation with the NVS they may be euthanised using a Schedule 1 method.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow any published guidance for large animals that may improve our study outcomes such as published by LASA or NC3Rs or the Home Office. We will ensure only the minimum number of animals are used as per trial design (pilot study or full experimental trial). The applicant routinely conducts power calculations on sample size (Genstat, VSNi Ltd) for all studies using a priori data.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The PPL holder is a member of an AWERB committee and regularly hears about updates on NC3Rs, from colleagues and the NC3Rs website. Any improvements will be implemented without hesitation. For example, in an alternative project, the PPL holder

altered the anaesthetic regime in consultation with the NACWO, named vet and veterinary consultants when it was apparent that a different regime produced better study outcomes. Equally, we sought to reduce unnecessary harm to sham-controls when it was apparent they were similar to other controls that had undergone full sham-surgery.

67. Studying neuron-glial interactions during nervous system formation, function and repair in zebrafish

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Brain development, Brain health, Multiple Sclerosis, Neuroprotection, Zebrafish

Animal types	Life stages
Zebra fish (Danio rerio)	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is use zebrafish to understand how nerve cells (neurons) and support cells (glia) of our nervous system develop and how their interactions ensure proper function. We will also use zebrafish to study changes to neurons and glial cells relevant to human conditions and to help find strategies to treat features of disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

At present we have a limited understanding of the complex mechanisms that underpin the formation and healthy maintenance of a functional nervous system.

This limits our ability to prevent or manage the effects of developmental and degenerative disorders of the nervous system, which represent major societal burdens. Part of the reason for our limited understanding of nervous system lies in the vast complexity of the brain and the many interactions between cells of the nervous system that are required to orchestrate its formation and function. About half of our brain cells are neurons, the electrically active nerve cells that drive communication across our nervous system and bodies. The other half of our brain cells are called glial cells, and there are different groups of glial cells with many different functions, which include providing nutrition to neurons, allowing our neurons to talk to our immune system or our blood vessels, and in supporting the function of our neurons. We will use the relatively simple zebrafish model to help discover how the brain is formed, how it functions, how it is disrupted in disease, and how we might develop strategies for its repair. We are particularly interested in studying the interactions of neurons and the glial cells that make myelin in our nervous system.

Myelin is a substance made by glial cells called oligodendrocytes in our brain and spinal cord that is wrapped around the electrical cables of our neurons. Myelin allows our electrical cables to transmit signals properly. Myelin is damaged or lost in many human diseases, including multiple sclerosis, and we need to find ways to help prevent myelin damage or loss, or to help regenerate myelin after it is lost. The zebrafish is a vertebrate with many similarities to humans. Zebrafish have many of the neurons and glial cells that we do, and the myelin-producing glial cells that we do. They also have an array of experimental advantages for studying neurons, glial cells and their various interactions the intact living nervous system.

What outputs do you think you will see at the end of this project?

The work of this project will teach us how nerve cells (neurons) and nerve supporting cells (glial cells) of the brain are formed, grow and interact to ensure normal nervous system formation, health, and function. We have a particular interest in how the nerve cables of our neurons grow to the right size, which is important for their function. We also seek to find out how neurons and the glial cells interact to ensure the proper firing of electrical impulses along nerve cables. Over the course of this project, we expect to learn how different neurons come to have nerve cables (called axons) of different sizes. We will also learn how the electrical activity of neurons influences both the axon and its myelin and the proper transmission of the electrical impulse along the axon. We will learn the identity of the signals that active neurons use to communicate with the myelin producing glia, the receptors that the glial cells use to listen to those signals and we will learn how this communication influences specific features of electrical impulse conduction, such as speed and timing, which are essential for behaviours.

We will also learn how neurons and glial cells react when we create zebrafish that model specific aspects of human diseases. For example, we will ask how neurons and myelin-producing glial cells respond when damage to and loss of myelin is induced. Damage to and loss of myelin is a feature of many human diseases and of advancing age. We can use zebrafish to directly visualise how cells respond to the induction of damage. For example, we will learn how glial cells repair damaged myelin and how neurons are affected by damage to or loss of myelin. We will also use zebrafish to carry out projects to

how to slow, stop or reverse damage to the nervous system. For example, we might identify a drug that helps myelin repair, or that stops neurons from degenerating after myelin has been damaged.

Our outputs will principally be the generation of manuscripts that summarise our findings.

We will ensure that we publish manuscripts in scientific journals open to the public. We will present the results of our studies at local, national and international scientific conferences and through appropriate media outlets. For example, we will prepare press releases and social media-based outputs to convey the findings of our work to the public in a digestible manner. In addition to the generation of new information, we will continue to make new genetically altered zebrafish that we will share with researchers around the world to accelerate their own related research studies. If we identify ways to slow, stop, or reverse nervous system damage, we will work partners at both academic and industrial institutions on therapy development projects. Our long-term goal is to help find treatments for developmental and degenerative disorders of the human nervous system.

Who or what will benefit from these outputs, and how?

Numerous groups will benefit from the outputs of our studies. The scientific community directly involved in studying mechanisms of brain development and disease will benefit from new knowledge that can be integrated into our growing understanding of the nervous system. Pharmaceutical companies will benefit from our research and our work could lead to bring us closer towards finding treatments for human diseases. Therefore, we hope that patient groups, families, carers and the wider community will ultimately benefit from our work, through the development of strategies to cure disease. During the course of our project the general public will benefit from knowing that progress is being made in tackling major areas of unmet need in human health.

How will you look to maximise the outputs of this work?

We maximise the output of our work by collaborating widely with groups who have diverse expertise in distinct technologies and model systems. We collaborate with both academic research groups and major pharmaceutical partners to help increase the impact of our work in so far as possible. We make our findings publicly available through open access publications. In addition, we present our work widely at local, national, and international meetings, and through various media outlets.

Furthermore, and importantly, we work closely with medical charities to convey our work to the public, and regularly contribute to information dissemination campaigns.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 115250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.
We use zebrafish as a model organism to study the formation and function of the nervous system, to assess the response to nervous system damage, and to find strategies to bring about repair.

Developmental and degenerative diseases of the nervous system represent a major burden to society, and there are currently very few treatments for disease. This is in part due to the complexity of the brain and in part due to the difficulty of observing key biological events in real time in most animal models. Furthermore, the feasibility and cost of carrying out large-scale discovery projects in most vertebrate models is prohibitive. We use zebrafish as a model to help overcome these challenges.

Zebrafish are vertebrates that exhibit remarkable conservation in terms of their molecular and cellular makeup with humans meaning that they often have shared mechanisms related to diseases and respond to gene and drug based manipulations in similar ways.

We primarily study young developing zebrafish in the laboratory, because they are small, relatively simple, develop quickly, building a functional nervous system in a week, are optically transparent, and can be generated in very large numbers. These features, together with our ability to create genetically altered animals, and treat young zebrafish with drug like compounds, means that we can directly see into the brain and observe brain development, brain function and even brain pathology as it occurs over time. This then allows us to investigate how experimental manipulations, for example drug treatments affects such processes in real time. Thus, the use of zebrafish allows us to gain insights into biological events that are difficult to achieve using other systems and to identify strategies to treat disease at an unprecedented scale.

Typically, what will be done to an animal used in your project?

The majority of animals that will be used in our project will be genetically altered zebrafish that have fluorescent proteins in cells or tissues of interest that allow us to track biological events in real time using a range of cutting-edge microscopes.

Many of the animals that we use in our project will also have genetic alterations that change the function of specific genes of interest, e.g. a gene associated with a human disease. In other cases, we make use of genetic manipulations to induce damage to cells or tissues that resemble those of diseases of the nervous system, or in other cases we use genetic manipulations that allow us to manipulate and study the function of cells of the brain. In many cases, we will also treat zebrafish with chemical compounds to assess how they affect the animal, and in particular the formation and health of its nervous system. In most cases, chemical compounds can be applied to the animals by diluting them in the water in which the animals live, but in a smaller number of cases we may have to use other approaches, such as injecting compounds directly.

The main experimental approaches that we undertake are a range of microscopical studies of genetically altered zebrafish. Almost all of the animals that we image by microscopy are under 3 weeks of age, because they are optically transparent at this stage and just a few millimetres in length. These properties mean that we can place such animals under a microscope in a drop of agarose (gel) and watch biological events as they happen over time. When doing so, and because we study animals at very high resolution to see fine details of the brain, we need to keep such animals still during microscopy. To

do so, we typically use anaesthetics to immobilise zebrafish, which can even allow long-term (over hours) time-lapse microscopy of events as they occur. In other rare cases we immobilise zebrafish in gel without anaesthetic or with a neuromuscular blocking agent, which we typically do to be able to study brain cell activity, which can be affected by anaesthetics. We also have a robotics-based microscopy platform that allows us to assess how treatment of large numbers of zebrafish with large numbers of compounds affects specific biological processes, e.g. the regeneration of brain cells in disease-like states. We have many years of experience of carrying out a variety of microscopical investigations on zebrafish, and the vast majority of our studies do not lead to adverse effects beyond mild and transient to the zebrafish. Because our interests lie in understanding the nervous system, some of our experimental manipulations, and very rarely our analyses, can lead to moderate adverse effects, for example changes to the swimming capacity of zebrafish. All efforts are taken care to minimise the chances of adverse effects being experienced. We do not plan any procedures that are considered to lead to severe adverse effects and none of our procedures require surgery, which highlights a great advantage of using the small transparent zebrafish as an experimental system to limit the potential adverse effects that the animal might experience. The various combinations of genetic alterations and chemical compound-based treatments that we will carry out will allow us to gain great insight into nervous system formation, function, disruption and repair.

What are the expected impacts and/or adverse effects for the animals during your project?

Due to the small size and rapid development of young zebrafish, and the fact that they can get nutrition from the maternal egg for the first week or so of life, we can carry out a large number of our experiments in non-invasive manners, and typically with little and often no signs of adverse effects on the animal. We are, however, interested in treating diseases of the human nervous system, and so we have created models of certain aspects of human diseases using zebrafish. These include systems to ablate cells of the nervous system, or the alteration of gene function that disrupts nervous system formation or function. In some cases, such animals can exhibit adverse effects including disrupted development or motor outputs. In the majority of cases, we can study animals with such adverse effects at stages prior to their being considered sentient enough to experience any suffering, and will make every effort to do so.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We expect about 50% of the animals used on this licence to exhibit sub-threshold effects, up to 45% mild adverse effects and up to 5% moderate adverse effects.

We do not plan to study animals that experience severe adverse effects.

We expect approximately 50% of all animals used under the remit of this licence to exhibit sub- threshold adverse effects. This reflects the fact that a large proportion of animals on this licence will be used for the generation, breeding and maintenance of genetically altered strains, and that approximately 90% of those animals exhibit no adverse effects.

We expect that up to 45% of all animals used under the remit of this licence could exhibit mild adverse effects. This number reflects the fact that animals with genetically alterations may exhibit mild adverse effects, such as subtle changes to behaviour, or that animals undergoing experimental protocols may experience mild stress due to being restrained during different forms of microscopy.

We expect that up to 5% of all animals used under the remit of this licence could exhibit moderate adverse effects. This is due principally to genetic alterations or cellular manipulations that disrupt nervous system development or lead to neurodegeneration and may be manifest as impaired motor outputs, such as disrupted swimming, or could be due to complex interactions of cells of the brain and body and be manifest in increased stress. We would only study animals experiencing moderate adverse effects for short periods of time. However, even brief analyses of zebrafish can be very informative, due to their rapid development and the ability to directly watch biological events in the animal in real time.

What will happen to animals used in this project?

- Killed
- Kept alive at a licensed establishment for non-regulated purposes or possible reuse

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The formation and function of neural circuits in the vertebrate central nervous system is incredibly complex. Brain development, function, and healthy maintenance involves intricate interactions between neurons, between neurons and glial cells and between these cells and our immune and cardiovascular systems. Many of these cellular interactions are not yet possible to study without animal models, because they are so hard to recreate in a dish. The consequences of damage or disruption to the nervous system also triggers very complex cellular responses and interactions that cannot easily be reconstituted without animal models. Therefore, to be able to understand how neurons and glial cells interact to construct the nervous system we need animal models and to see how these cells are influenced by the immune system and vasculature in disease, it is also currently essential to employ animal systems. However, in using zebrafish, we make use of arguably the simplest vertebrate model in which the complexity of the nervous system can be directly interrogated.

Which non-animal alternatives did you consider for use in this project?

Certain aspects of neuron and glial cell development, and even certain aspects of myelination can be studied without using animals, by studying cells grown in the dish. We work closely with colleagues who have expertise in studying cells in the dish and we have gained many insights from such work.

However, this current project aims to use the simple zebrafish model to begin to study the complexity of brain formation, function and disease as it occurs in the natural setting. At

present there are no other suitable non-animal alternatives to do so. We considered using cell culture techniques that now allow mini-brain-like "organoids" in the dish, and are hopeful that in years to come technologies will become so refined that we they can be used to study neuron and glial cell interactions and neural circuit function. Such cell culture systems may even allow us to model certain aspects of disease in the dish, and we look forward to incorporating such models into our work.

Why were they not suitable?

Brain organoids are beginning to show great promise as an experimental tool for neuroscience, but have not yet been established to the point where they have all of the neurons and glial cell types that would be required to be able to probe the mechanisms of nervous system formation and function.

Furthermore, in the context of modelling disease, brain organoids have not yet been developed with a vascular system and the full complement of immune cells that are known to influence pathology and repair.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have calculated the number of animals that we are likely to use based on our current use of zebrafish as a model system. One of the major advantages of using zebrafish as an animal model is that many distinct genetically altered lines of zebrafish can be maintained by single laboratories and that the system can be used for large-scale discovery projects that are not feasible using mammalian models. Therefore, we plan to use a large number of animals through our project. Of the animals we project to use, that majority are for breeding and maintenance purposes. We have calculated the projected number of fish, based on our current use of approximately 500 tanks of zebrafish per week, in which we keep an average of 20 fish. We aim to refresh stocks once per year, meaning that we will use 5 separate generations of each stock over the course of the project, giving a total of 50,000 animals. We maintain >150 distinct genetically distinct lines of fish, many used by several users, and constantly monitor our stocks to ensure that we are not maintaining lines that are not in use. Over the next years, we plan to cryopreserve novel stocks not in active use, so hope that we reduce the number of animals maintained from this initial number calculated.

We also expect to use of up to 15,000 zebrafish for the generation of new genetically altered animals. This number largely reflects the ability to assess the effect of altering gene function in animals directly after injecting reagents (especially CRISPR-cas9) that can edit the genome. With zebrafish, we can look for the effects of disrupting gene function within days of such "gene editing" of individual animals.

The ability to target gene function directly means also that we can quickly assess how individual genes affect many different biological functions. For example, we can see how editing different genes affects different cell types or disease states because we can directly edit genes in animals with fluorescent reporters in their neurons, glial cells, immune cells, cardiovascular system etc. Testing the effects of 200 genes over the course of this project in 3-5 assays using 10-15 animals per assay will require 10,000 animals.

Following such tests, we will create genetically altered lines for the genes exhibiting the most important roles in regulating nervous system formation, health or function.

We would plan to test up to 20 genes in this way by creating stable genetically altered lines. To identify the animals that we use to propagate stable lines for further breeding and maintenance, we have to test up to 50 potential founder fish per line, using a total of 1000 fish.

In addition we will make additional new genetically altered lines, e.g. that employ transgenic technology to label cells or proteins of interest, create specific models of human disease, such as introducing a human gene in place of a zebrafish gene, or by generating genetic alterations in specific cells only. We expect to create 20 new lines in this way, which can necessitate testing up to 200 potential animals per line for successful genetic manipulation, using a total of up to 4000 fish.

In addition to the 10,000 gene-edited animals that we will study directly after their gene editing, we predict using a further 40,000 animals in our experimental analyses.

This is driven in large part to our success in establishing screening systems that allow high-resolution imaging-based analysis of larval zebrafish anatomy or behaviour. Using this system, we can screen how drug-like compounds, as well as the genes we edit, affect up to 500 fish per day. Although we anticipate that many of our studies will continue to be carried out at embryonic stages (5 days after egg fertilisation and under) before zebrafish are considered sentient enough to require legislative protection, we have established transgenic models in which we can ablate myelin, i.e. cause demyelination, which we study after 5 days of age. With this demyelination system, we can use our screening system to search for drug-like compounds that can, for example, promote myelin regeneration or reduce neurodegeneration. We anticipate using up to 30,000 animals in our screening system, and have predicted using a further 10,000 for other experiments, based on our use over recent years. We carry out very careful calculations to define how many animals are needed to find statistically meaningful effects in our experiments, and will continue to do so for new studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The principal step that we take in our experimental design is to determine what questions we can address using zebrafish at unprotected stages, i.e. early developmental stages prior to 5 days post- fertilisation of the zebrafish egg, when the nervous system is considered to be in an immature state such that the animal does not to have the capacity to suffer. Nonetheless, the nervous system at this stage does contain nerve cells and glial cells that can interact with one another, allowing us to study fundamental biological events in an intact system. Because of our ability to study certain biological mechanisms at these stages, we can significantly reduce the number of animals that we use at more mature and

thus protected stages on experimental protocols, and we will continue to pursue this strategy. However, the modelling of disease and the analysis of some aspects of neural circuit maturation are not possible to carry out at unprotected stages, nor are breeding and maintenance protocols, nor the generation of new stocks of genetically altered lines.

Nonetheless, we can assess the efficiency of transgenesis and gene editing at unprotected stages, which reduces the number of animals taken on to protocols.

Another important way in which we can reduce animal number is through live imaging of individual animals over time. Through time-course or time-lapse imaging, we can gain a wealth of information about the dynamic nature of biological events from single animals that would otherwise require multiple animals being assessed at many different time-points.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will continue to work to optimise the efficiency of transgenesis and gene editing, particularly cell- type specific gene editing, which we hope will reduce the number of animals that we use in our work. We will also work closely with our aquarium staff who are implementing trials that aim to adapt husbandry procedures to ensure more reliable sex ratios in our breeding stocks. Skewed sex ratios are prevalent in zebrafish stocks and definitive protocols to balance male and female generation have not yet been established. This would help reduce the number of animals that we need to use to generate sufficient breeding stocks for our experiments. In many cases, we will also be able to carry out pilot experiments on zebrafish that can be shared by other users who have the authority to do so.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use zebrafish to study nervous system formation, function, disruption and repair.

We use zebrafish due to the ease with which one can generate and maintain genetically altered animals with fluorescent reporters that allow direct visualisation of molecules and cells of the nervous system in embryonic and larval stages without the need of invasive procedures, and typically without any evidence of adverse effects. We also use zebrafish because of the ease of gene editing and expression.

Again, the ability to carry out transgenic manipulations and gene editing on newly fertilised eggs means that their efficiency and any potential adverse effects can be observed prior to

their development to ages that are protected. This is an important refinement that reduces animal numbers used and helps reduce any potential suffering the animal might experience, were it not possible to do so.

Our principal experimental methods involve live imaging zebrafish, and we can do so at different scales. We can carry out high-resolution screens of many animals to quickly assess how gene or compound function affect biological processes of interest, and in a manner that shows no signs of causing distress to the animal. In contrast we can also carry out extensive in depth imaging over time of individual animals, either of the structure or function of their nervous system in a healthy or disease context. Time-lapse or time-course imaging of individual animals is a refinement, because we can gather enormous amounts of information from single animals. Such analyses provide insight into dynamic processes impossible to gain in other systems that would require the use of multiple animals if using other models.

We continue to pursue any innovations in husbandry practices, and when trialled and deemed successful, will be applied to our protected stocks, and may significantly reduce numbers of animals used for breeding and maintenance throughout the project.

Why can't you use animals that are less sentient?

We are particularly interested in interactions of neurons and glial cells that orchestrate nervous system formation and healthy function. Glial cells are generally generated after neurons and as functional neural circuits mature. Therefore, their analysis cannot be carried out at very early/ embryonic life stages. Many glial cells are specific to vertebrates. For example, the glial cells that make myelin of the central nervous system, oligodendrocytes, are not found in invertebrate animals, and so such models cannot be used for their interrogation. However, zebrafish are arguably the simplest standard laboratory model in which glial cells and their interactions with neurons can be studied.

Furthermore, the zebrafish nervous system develops rapidly with an array of glial cells formed and functional in animals of just a few days old. For example, zebrafish have an early onset of myelination and at stages that one would reasonably expect their being less sentient than mammals by the time they undergo myelination at postnatal stages. In all of our work, we make every effort to study animals at the earliest stages at which we can address the questions that underpin the aim of our studies, to minimise the likelihood of animals experiencing adverse effects due to our work.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Very few of our experimental protocols cause significant harm to animals and are already well refined. The main source of potential adverse effects to animals comes from the generation of new genetically altered animals where all possible effects on the animal are not possible to predict. However, as noted throughout, we can assess how new genetic alterations affect animals at unprotected stages and we carefully monitor animals following the introduction of new genetic alterations. We have recently implemented a new stock management database in our facility that allows us to better track and monitor survival rates and any effects seen across all of our stocks.

What published best practice guidance will you follow to ensure experiments are

conducted in the most refined way?

We follow the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines issued by the National Centre for the 3Rs, and will follow the recently published guidelines put together by a group of zebrafish researchers in collaboration with animal welfare experts at the Federation of European Laboratory Animal Science Associations (FELASA). In addition, we continue to refine practice across all experimental approaches as innovations and advances are published in the literature.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

My group and I follow NC3Rs on social media, and stay informed about relevant innovations through our proactive NIO at the University. We will implement appropriate advances through discussions with our local vets and named animal care and welfare officer.

Our establishment has a departmental website and sharepoint site for staff conducting research with protected animals and includes sections relevant to the 3Rs and relevant species specific information. Our establishment also subscribes to Norecopa which has a range of excellent resources related to promoting and implementing the 3Rs in Zebrafish research https://norecopa.no/species/fish/zebrafish/.

In addition, we will continually work with collaborators and other experts in our field to share expertise and experience with refining relevant experimental models.

68. Models of bone disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Bone loss, Post-menopausal osteoporosis, Steroid-induced osteoporosis

Animal types	Life stages
Mice	Adult
Rats	Adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to identify new medicines to treat bone disorders, such as post- menopausal or steroid-induced osteoporosis, and assess the impact of aging on efficacy of these potential novel therapeutics.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Loss of bone mass may be caused by a variety of factors in patients, although two predominant reasons include the reduction in circulating oestrogen levels following the menopause in women, or treatment with certain steroid drugs, which are commonly used to treat chronic inflammatory conditions. Bone mass typically reaches peak levels early in adult life (~ early 20's) and loss of bone mass is unfortunately an inevitable aspect of

aging, particularly in women, where bone loss rapidly accelerates following the reduction in oestrogen levels following the onset of the menopause.

Oestrogen is a powerful survival factor for the cells of the body that make bone (osteoblasts), and therefore the reduction of oestrogen following the menopause negatively affects bone mass. Similarly, certain steroid drugs such as prednisolone and hydrocortisone can negatively affect the delicate balance between bone formation and bone breakdown in the body, leading to an overall loss of bone mass that may occur following short-term treatment with high-doses of such drugs, or as a result of long-term treatment for chronic inflammatory conditions.

Definitions of low bone mass are derived based on mathematical calculations of normal bone mass found in general populations, which is described by a so-called T score.

Severity increases from reduced bone mass, termed osteopaenia (T score of -1 to -2.5), up to the more severe classification of markedly reduced bone mass, termed osteoporosis (T-score >-2.5). Fragility fractures occur in response to a fall or other such injury, where the loss of bone mass has reduced the mechanical properties of bone to a level where they are unable to resist fracture. As such, fractures involving the vertebrae and at non-vertebral sites (e.g. forearm and hip) markedly increase in osteoporotic patients, which, in the case of the hip and vertebrae, are associated with dramatic increases in morbidity and mortality, particularly in the elderly population.

Whilst there have been numerous advances in the management of bone loss in recent years, such as the anti-resorptive drugs (bisphosphonates and denosumab) and the bone-forming drugs (e.g. parathyroid hormone derivatives or romosozumab), there remain issues with these drugs, such as lack of efficacy, or troublesome side effects, in a significant number of patients. Furthermore, there remains an urgent clinical need for safe and effective agents capable of reliably inducing bone formation, to reduce the risk of bone fracture in aging populations. This project will aid in the development of novel therapeutics to enable better treatment of patients currently living with these diseases.

What outputs do you think you will see at the end of this project?

This project will allow a better understanding of the biological mechanisms underlying bone loss in disease and suggest novel approaches for treating patients.

The output will include identification of new therapeutic candidate molecules, which can then move forward into clinical trials.

We will aim to publish and disseminate novel findings at conferences in the form of posters and oral communications and also in peer-reviewed journals.

Who or what will benefit from these outputs, and how?

Ultimately, the beneficiaries of the work performed in this licence will be patients with excessive bone loss due to osteoporosis. Typically, novel therapeutics take an average of 10 years from conception through clinical development and into market for patient benefit.

In the shorter term, there will be benefits to scientific research community through dissemination of new protocols, methods and publications developed during the project.

How will you look to maximise the outputs of this work?

We will seek to build on existing collaborations but also engage with further key opinion leaders in the relevant academic fields and industry, to further advance our common understanding and accelerate the development of new therapies. We will also aim to present our key findings and results at academic conferences and to publish in peer-reviewed scientific journals.

Species and numbers of animals expected to be used

- Mice: 3000
- Rats: 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Bone is a complex tissue which is subject to regulation by numerous mechanisms including cells of the immune system and also mechanical stimuli. For example, bone homeostasis is controlled by mechanical sensing by the osteocyte, which then signals to the osteoclast and osteoblast to either resorb or build bone, respectively.

Consequently, no in vitro system currently exists to faithfully reproduce such complex regulatory mechanisms, thereby requiring in vivo systems to capture the complex biology underlying diseases involving bone.

Rodents are the mammalian species of lowest neurophysiological sensitivity in which these bone disease models have been investigated and shown to replicate human disease. In this application the species listed include mice and rats and the choice of species in a given protocol will depend on published studies and known similarities of target gene expression between rodents and man. The use of genetically altered (GA) mice will also allow the study of specific genes involved in bone homeostasis and also in disease.

Typically, animals will be used at skeletal maturity (typically 10-12 weeks), in line with most published studies involving bone studies in mice and rats in the scientific literature. To maximise the relevance of such studies in the context of human disease we will also study older mice (up to 12 months of age; when age-induced bone loss becomes evident in specific mice strains, e.g. C57Bl/6), to determine whether changes identified in younger mice are also reflected in more mature animals.

Typically, what will be done to an animal used in your project?

Typically, rodents used in this study (minimum 10-12 weeks of age) would fall into 2 groups: healthy animals with normal bone mass, and animals with reduced bone mass. Low bone mass would be induced pharmacologically, e.g. using sustained release corticosteroid administration, or via surgical models, e.g. ovariectomy (Ovx), to mimic bone

loss associated with postmenopausal osteoporosis (Ovx animals would be purchased from licenced providers).

Into each of these backgrounds (normal, or low bone mass) a therapeutic substance would then be administered - typically using standard routes e.g. intravenous, subcutaneous or oral.

The impact of this treatment would then be assessed by daily weighing and welfare scoring sheets used to monitor animal well-being. Blood sampling would be performed to allow readouts of bone relevant biomarkers (such as PINP or TRAcP, as markers of bone formation and resorption, respectively) over the course of the treatment period. During the study, longitudinal assessment of bone formation would be performed using in vivo microCT scanning performed under general anaesthesia (typically 25-30 min scan duration, performed at regular intervals - typically 1-2 weeks apart). Bioluminescent imaging (IVIS) may also be performed in a subset of experiments, also under general anaesthesia.

At the end of each experiment all animals will be humanely killed, by a Schedule 1 method.

All procedures have been ethically reviewed and all animals undergoing procedures will be well looked after by trained staff that work closely with a veterinary surgeon.

What are the expected impacts and/or adverse effects for the animals during your project?

Ovariectomised animals, purchased from licensed vendors, will typically be supplied no sooner than 4 days following Ovx surgery, thus may suffer from moderate pain or discomfort as they recover from the procedure. Once arriving on site, they will be assessed and allowed to acclimatise for a minimum of 7 days prior to administration of any test compounds.

Pharmacologically-induced bone loss would be achieved via subcutaneous implantation of slow- release pellet formulations of prednisolone, for example, to minimise the necessary number of repeated injections previously required for such model systems. Typically, such models in rodents are associated with minimal changes to body weight, appearance or behaviour. Bone loss will then be confirmed by in vivo microCT scanning (Bruker Skyscan), which requires general anaesthesia for a scan duration of approximately 25-30 mins and limited radiation exposure to the scan site (e.g. distal femur or proximal tibia), previously shown to have minimal impact to the biology of the scan site.

Blood sampling may also be performed in mice or rats via tail vein sampling (subject to the standard limits on volume for the duration of the study) to allow monitoring of changes in bone turnover markers but this will cause only minimal discomfort to the animals. Test compounds may be administered either via dietary administration, oral gavage or s.c. injection, which should produce only temporary discomfort and minimal changes in behaviour.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per

animal type)?

30% of animals (rats and mice) will experience a moderate severity. 70% of animals (rats and mice) will experience only mild severity.

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The processes of bone formation, remodelling, and repair from damage are driven by a complex interplay between multiple cell types and a variety of mediators, together with a further layer of complexity provided by the important role that mechanical loading plays in regulating bone mass. Although advances are being made in more complex cell culture models to incorporate, for example, multiple cell types together with mechanical loading, it remains challenging to recreate these processes solely via the use of in vitro cell assays and/or computer modelling approaches.

In addition, in vivo data on the pharmacological activity and efficacy of novel drugs is required by the regulatory authorities for the development of new medicines.

Which non-animal alternatives did you consider for use in this project?

Cell culture and organ-on-a-chip (OoC) technologies may model some individual disease mechanisms, and these will be used wherever possible. An up-to-date knowledge of the literature and latest technologies will be gained from consulting resources such as:-
Fund for the Replacement of Animals in Medical Experiments, FRAME (https://frame.org.uk/); Animal Welfare Information Centre (https://www.nal.usda.gov/programs/awic);
EU Reference Laboratory for alternatives to animal testing (EURL ECVAM) (https://joint-researchcentre.ec.europa.eu/eu-reference-laboratory-alternatives-animal-testing-eurl-ecvam_en).

Why were they not suitable?

A combination of multiple mechanisms and cell types are required to satisfactorily recapitulate the processes involved in bone formation and bone loss, which are not currently replicated in in vitro systems.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise

numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These numbers are based upon the typical n numbers required in for an individual experiment, with appropriate control groups and theoretical treatment regimens of candidate therapeutic molecules. This number refers to the total number of experiments expected to be performed during the entire 5-year duration of the project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our statisticians are regularly consulted regarding appropriate sizing for our proposed experimental groups and appropriate statistical tests for our experimental data.

Appropriate group sizes will be based on power calculations and estimated effect sizes for candidate therapeutics, to appropriately identify any statistically significant differences between the groups whilst minimising the numbers of animal usage. The group sizes are under constant review, taking into account the experimental variability and intra-group variability in a variety of assessment criteria.

Wherever possible the effect of a therapeutic will be determined on multiple readouts from a single experiment, thereby reducing the number of animals used overall. In addition, the use of longitudinal in vivo microCT scanning (or other bioimaging modalities, e.g. IVIS), whereby individual animals are scanned at regular intervals over time, will minimise animal usage by reducing the need for culling animals at singular time-points and reducing experimental variability (by using each animal as its own control for baseline assessment).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

A significant proportion of drug discovery is carried out using cells and cell lines using in vitro assays, with potentially thousands of candidate molecules being screened to identify the most promising compounds/antibodies. To ensure the fewest number of animals are used, only the most effective compounds that have been pre-screened for activity in vitro will be subsequently brought forward for testing in animals. Only a limited number of these compounds/antibodies will be evaluated in this project.

An initial pilot study consisting of a single high dose group plus control may be conducted initially to determine efficacy prior to larger multi-dose group studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why

these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

In this project we will assess novel bone therapeutic molecules in healthy animals and also in animals with a low bone mass phenotype (i.e. osteoporosis) induced either via surgery (ovariectomy; Ovx) or pharmacologically using corticosteroid administration. These approaches are well established models in the scientific literature, with only limited detrimental effects on animal welfare once surgical recovery from Ovx has occurred. Bone loss in both model systems occurs over a number of weeks and is typically not associated with any overt signs of pain, suffering, distress or lasting harm within the planned experimental duration. Animal welfare scoring sheets will be employed to help monitoring of the animals and to confirm that no detrimental issues occur during the study of either healthy animals or those with low bone mass phenotypes.

New molecules will have undergone extensive in vitro profiling prior to in vivo testing.

Furthermore, pharmacokinetic and tolerability studies will also have been performed on any NCEs prior to use in this study to ensure that suitable dosing regimens and durations are determined to produce the required exposure to show in vivo efficacy.

In our experience, adverse drug reactions have generally been found to be rare (<1%) with the assistance of in vitro screening in the identification of molecules that are likely to be toxic.

Why can’t you use animals that are less sentient?

Rodents (rats and mice) are the mammalian species of lowest neurophysiological sensitivity in which these models have been developed and retain relevance for human bone physiology.

High bone formation rates in rapidly growing neonatal and juvenile animals typically preclude their use in such studies of bone therapeutic efficacy, with the majority of studies in the scientific literature being performed on mice and rats at 10-12 weeks of age, an age more closely associated with skeletal maturity. The use of more mature animals (up to 12 months old) will also allow the testing of therapeutic efficacy in a more relevant aged system, given the greater clinical relevance of increased age to osteoporosis incidence in humans.

Generally, these assessments of therapeutic efficacy require such models to run for a minimum of 4 weeks, thereby precluding the use of terminally anaesthetised animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animal welfare scoring sheets are used to monitor and limit the severity of any observed adverse events. Strategies to minimise the number of needlestick injuries, such as those required for drug administration, for example, will be minimised wherever possible, based on the use of slow-release pellet formulations (as with prednisolone to induce the low bone mass phenotype) or by attempting to administer test compounds via dietary or oral administration.

Given the robust and widespread use of the proposed surgical protocols (Ovx and subcutaneous prednisolone pellet implantation) in the field of bone biology, we anticipate that there would be limited opportunities for further refinement. However, where improvements to pain management practices or surgical techniques are available they will be incorporated where possible. Pain management and best practices associated with the necessary use of general anaesthesia during microCT scanning will be optimised with the help of our Named Veterinary Surgeon.

Humane endpoints are employed to limit animal suffering.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will continue to review and learn from relevant literature to ensure our models incorporate any new advances in our 3Rs strategy.

We will regularly consult with the Resource Library on the nc3rs.org website to ensure best practices are performed. Furthermore, we commit to using the PREPARE guidelines for planning in vivo studies and the ARRIVE guidelines for reporting our findings in resulting publications.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Members of our Special Facilities Unit and members of our in vivo pharmacology team routinely attend conferences such as LASA, FELASA, and conferences run by the RSPCA to discuss advances in 3Rs and to bring to our attention new learnings or strategies that can be implemented in the project. These may be through simple changes or periodic amendments to the licence to better reflect best practice and improved guidelines for conducting in vivo studies.

69. Hay as a vector for scrapie transmission in sheep

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

scrapie, prion, environment, plant, contamination

Animal types	Life stages
Sheep	Juvenile, Adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This study aims to determine whether hay can act as vector for transmission of the scrapie agent, which causes a transmissible spongiform encephalopathy in sheep.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Scrapie, a transmissible spongiform encephalopathy (TSE) in sheep and goats, is caused by prions, proteinaceous infectious particles, which are extremely resistant to biological inactivation. They are known to remain active in soil for decades and have been found in plants and roots, either because prions are taken up via soil or because secretions from infected animals have contaminated plants directly. It has been shown in an experimental mouse model that plants can act as vectors for scrapie transmission, but it is not known whether this could happen under natural conditions. It has also been shown that sheep

grazing on pastures that were previously used by scrapie-affected sheep can be infected, but the source of infection has not been established: plants, soil ingested with plants, contaminated field furniture like water troughs, feeding stations or fences, or a combination of all of these. If plants harbour prions based on testing fresh material, it is unknown whether the drying process (by producing hay) will reduce infectivity to a level where feeding it to susceptible livestock will no longer cause infection, which may be the only option to feed plant material to livestock if the land is considered to be contaminated. Results from this study is imperative for our understanding of the epidemiology of prion diseases, which has become more important as cases decline in the UK, but the source of infection needs to be established whenever there is a new outbreak and actions need to be taken to prevent further spread or re-infection.

What outputs do you think you will see at the end of this project?

The results will be published in an open access peer-reviewed journal and - if not already picked up by the farming press - the information will be summarised and provided to relevant stakeholders that need to know whether hay is a source of infection.

Who or what will benefit from these outputs, and how?

The output will be relevant to farmers and land owners and not only the research community including epidemiologists and risk assessors, as it will provide immediate information about the "safety" of hay from prion-contaminated pastures. It is also relevant to protect our borders from introducing diseases into this country via plants, particularly chronic wasting disease, which is an exotic prion disease in deer. The timeline of this depends on the outcome; if the level of prions is very low or no transmission occurs it may take several years to obtain a positive result or confirm a negative result because of the long incubation period of prion diseases.

How will you look to maximise the outputs of this work?

The study will be published regardless of outcome as even a negative finding is useful for risk assessments. Any scrapie-positive animal will be culled to harvest tissue for storage that can be used for diagnostic purposes (e.g. as positive control material) or test development purposes. It is anticipated to retain some hay for *in vitro* prion detection tests to see whether these could replace sheep or other animal experiments in future (e.g. if there is agreement between animal and *in vitro* test with regards to positive or negative results).

Species and numbers of animals expected to be used

- Sheep: 14

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This study requires live animals to mimic the natural situation. Mice were used in an already published study but it was acknowledged that "aspects of these experiments that do not faithfully recapitulate the diseases they model include animal digestive tract structure (viz. mouse versus ruminant)". The study requires animals to be capable of eating hay but at young age as susceptibility decreases with increasing age, which is why it is proposed to utilise lambs that have just been weaned.

Typically, what will be done to an animal used in your project?

Animals will be fed hay as libitum, which may cause scrapie if it transmits prion disease. To determine infection, a rectal biopsy will be taken 6-monthly under local anaesthesia and examined for prions. A repeat may be required not earlier than 2 weeks after the initial biopsy if the biopsy did not yield lymphoid tissue for a diagnosis. Animals will be kept for at least 36 months post exposure if there is no evidence of infection based on biopsies (at least 5 biopsies will be taken if animals are culled at 36 months). A blood sample will be taken for diagnostic purposes at the same time as the biopsy and prior to euthanasia. Animals may either be culled when they have prions in the biopsy and thus will not develop clinical disease as detection generally precedes clinical onset or will be transferred to a different project licence if they are required to develop clinical disease to collect more tissues harbouring prions.

What are the expected impacts and/or adverse effects for the animals during your project?

We do not expect any relevant side effects for the rectal biopsy and blood sampling as these will only cause transient pain or discomfort associated with restraint and insertion of a needle or speculum (biopsy will be done under local anaesthesia). Sheep should not experience signs of disease under this licence.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild in all animals.

What will happen to animals used in this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Ultrasensitive *in vitro* tests have been developed to detect prions at extremely low

concentrations. They have demonstrated that plants can harbour prions using artificial settings, such as spraying plants with brain homogenates and subsequently testing them. We intend to use ultrasensitive tests in parallel but regardless of outcome the findings will be difficult to interpret: if positive by the *in vitro* test, is it applicable to the natural situation, i.e. would sheep become infected or is the amount so low that it is not biologically relevant? If negative, could this be because the amount is below the detection limit but may cause disease in sheep due to repeated exposure or is it because we used a sample that may not contain prions whilst sheep can consume a whole batch of hay?

Which non-animal alternatives did you consider for use in this project?

Ultrasensitive *in vitro* prion detection tests are used more and more frequently in prion research to detect prions. We intend to use them in parallel to establish whether they could be used as a replacement for future studies, e.g. to evaluate if pastures where deer graze and are used for hay production could be assessed for contamination by these tests.

Why were they not suitable?

Any *in vitro* test detects prions, which is different to infectivity. Basically, tissues may harbour prions (as detected by these tests) but they are not infectious when inoculated into animals or, conversely, prions may not be detectable even if the tissue is infectious. The contamination of plants may not be uniform, thus picking the right plant for the *in vitro* assay may be important for any correct interpretation. This is not a concern when the plants are fed to animals because many plants will be consumed.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Sample size is dependent on estimated rate of transmission (more needed if transmission is expected to be low), availability of hay (collected from a pasture with the possibly highest contamination as it was used by clinically affected sheep; the number of sheep will determine how long it will take to consume all hay), available accommodation (biosecure environment) and available sheep for the proposed time period (need to be highly susceptible but highly susceptible sheep are rarely available because of the National Scrapie Plan that led to an considerable increase in resistant sheep). A sample size of 14 was considered to be achievable in practical terms whilst providing enough confidence in the results, even though it may mean that the study has to be done in stages if not enough sheep are available in one year. In addition, susceptibility to scrapie reduces with age and animals over 12 months of age may not get infected, thus we aim for a maximum of 14 sheep to have consumed all available hay before they become "resistant".

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

As stated, estimated transmission rate influences the number of sheep to be used. We do not know whether sheep become infected at all but if the infection rate was high (e.g. 100%) we would only need animals in single figures (more than one to account for potential losses due to intercurrent diseases), which would consume potentially contaminated hay over a longer period of time than a larger number of sheep. We do not expect the infection rate to be as high as 100%, which is why we estimated the number based on what was practically feasible with a potentially low transmission rate, using an online sample size calculator.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

If transmission was demonstrated in the first year, starting with less than 14 lambs, there would be no need to expose more lambs in subsequent years.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This study will use sheep as natural host for scrapie. It is not known whether the sheep will actually become infected (no disease will occur) and if it is confirmed by rectal biopsy that they are infected, they will not progress to clinical disease under this licence because it is not needed.

We use rectal biopsies to determine scrapie status because peripheral lymphoid tissue is needed for a diagnosis in a live animal; otherwise we would need the sheep to develop clinical disease, euthanise them and then test the brain for scrapie. Most lymphoid tissues are not accessible in live sheep; other options are palatine tonsil biopsies (requires general anaesthesia with more side effects; less suitable for repeated sampling) or third eyelid biopsies (requires at least sedation and unsuitable for repeated sampling). These will be done 6-monthly to limit frequency but to detect an infected sheep early enough to remove it from the group and prevent horizontal transmission to others. We will not cull the remaining biopsy-negative sheep if one sheep is biopsy-positive to have some idea about the rate of transmission.
Blood sampling will be at the same interval as the biopsy and prior to euthanasia; not more than 50 ml will be collected 6-monthly and the largest volume collected may be prior to euthanasia, which will be via intravenous injection, so will not involve any additional needle stick.

Why can't you use animals that are less sentient?

Transmissible spongiform encephalopathies are slowly progressive diseases, with incubation periods lasting for months to years. We do not expect infection to be detectable in rectal tissues within 6 months after first exposure. As hay is fed we need sheep with a functional rumen to digest the hay, i.e. they need to be weaned (from 3 months of age).

Alternatives are mice, which cannot consume the same amount of hay as a sheep (problematic if the level of contamination is extremely low) and have a different digestive system compared to ruminants. For similar reasons, we do not propose to use transgenic drosophila flies, which will need to ingest hay in the larval stage. Whilst they may confirm infectivity of plants (if positive), the findings cannot be extrapolated to sheep as natural host.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be monitored for signs of scrapie by experienced animal husbandry staff and - if suspicions arise - will be clinically examined but we do not expect the sheep to develop signs of disease under this licence because prions will accumulate in rectal biopsies many months before onset of clinical disease. A clinical scoring system is used to detect early signs of scrapie, which has been refined over time because of the variation in clinical signs.

The volume of blood when or if collected will be below the allowed limit (estimated 60 ml/ kg, not more than 10% can be collected safely at a single bleed, which would be 240 ml for a 40 kg lamb). Rectal biopsies will be carried out under local anaesthesia using topical cream applied to the rectal mucosa. Both procedures will only be performed by trained and competent staff to avoid multiple needle sticks or inadequate biopsies.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the guidance from various sources (NC3R, Norecopa for procedural aspects; peer- reviewed publications or prion conferences for TSE-related tests).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

This is a long-term study and once started it will not be ethically justifiable to replace sheep or terminate the experiment prematurely. We will be kept informed about new developments in prion research, including similar studies in other species, through attendance of conferences or discussion with prion researchers. This includes also development of tests that may detect infection in blood rather than rectal biopsies, none of which are currently validated for use in sheep.

70. Understanding mechanisms of injury and repair in demyelinating disorders of the nervous system

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

central nervous system, glial cells, extracellular matrix, white matter injury, renin angiotensin system

Animal types	Life stages
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this program of work is to discover and develop new therapies for the treatment of diseases and conditions in which there is white matter damage in the central nervous system, e.g., such as occurs in the condition multiple sclerosis.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

In neurological diseases, such as multiple sclerosis, it is of the upmost importance to promote neuroprotection and to facilitate repair of the nervous system. Multiple sclerosis is a chronic neurological disease affecting over 3 million individuals worldwide and with an

estimated incidence of 130,000+ people in the UK. Current treatments for multiple sclerosis target modulation of the immune response. Whilst this is effective in part by reducing the number of relapses, unfortunately people with multiple sclerosis, still progress with disability suggesting neurodegeneration. It is important that new treatments are developed that can either promote protection of the nervous system in multiple sclerosis or stimulate the repair response.

What outputs do you think you will see at the end of this project?

Following injury to the brain and in diseases such as multiple sclerosis, important brain cells that make a fatty substance that covers and protects nerve cells, called oligodendrocytes, are damaged and die. When this happens, it can impact the functioning of the nerve cells they are protecting and can lead to further loss of function and death of the nerve cells. The events that lead to oligodendrocytes and nerve cells dying are still undetermined, as are therapies that can promote protection of these cells and approaches to enhance the restoration of function. At the end of this project, we will have gained new information as to how events and processes activated within the body can contribute to disease worsening and how we can use these pathways to promote protection and repair in the nervous system. We also hope to demonstrate therapeutic interventions by developing pre-clinical efficacy data using mouse models that replicate some of the damage observed in diseases such as multiple sclerosis. We will disseminate this new information through presentation at research meetings and through publications in peer-reviewed journals. In the future (beyond this project) it is hoped that by understanding these processes and demonstrating proof of concept experiments this will lead to the use of new therapies to promote nervous system protection and repair in white matter diseases.

Who or what will benefit from these outputs, and how?

In the short-term these outputs will directly benefit all researchers within the field including those from academic institutions or from pharmaceutical industries around the world. The future outputs from this research will shed new insight and has potential to impact future treatment interventions for people with multiple sclerosis and aid in clinical trial design through understanding the key events and pathways targeted during 1) disease pathogenesis and 2) therapeutic intervention .

How will you look to maximise the outputs of this work?

We can maximize these outputs by publishing our findings in open-access journals, presenting them at scientific meetings and making our resources available to other researchers (e.g., data, animals, tissues) as well as presenting to and attending patient focused groups.

Species and numbers of animals expected to be used

- Mice: 3800

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures,

including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use mice in our study in two ways. Firstly, we will use mice to obtain tissue from specific body areas, such as from the nervous system, that can then be grown in a dish. In our second approach we will use adult mice as a whole-body model so to study aspects of human disease processes. Typically when obtaining nervous tissue from the body to grow in a dish we will use embryonic stage mice, and to model disease in the whole mouse we will typically use adult mice (aged 8 weeks plus). These approaches allow us to understand either the processes activated in a reduced, simplified system or within an intact biological system and all the cells that are involved in these processes. These approaches allow us to determine how all events are coordinated together.

Typically, what will be done to an animal used in your project?

To harvest tissue to grow in a dish, no procedures, other than humane euthanasia (using schedule I approved killing methods) will be performed on the mice.
To model disease we will feed adult mice with a dietary toxin for example 3 to 6 weeks in duration and then study the brains of these mice for example 3 to 6 weeks after stopping the diet (example, up to 12 weeks total duration for the experiment). During this time we may also give mice a therapeutic treatment either by daily injections under the skin or by implantation of very small osmotic minipumps under the skin under general anaesthesia (these allow injections through the pumps negating the need for regular injections under the skin). For mice that are fed dietary toxin, the toxin may be mixed into the regular mouse diet, for which mice have free access to. After consuming the toxin for 3 to 6 weeks, the mouse diet is returned to normal feed, which again, mice have free access to. During this entire experiment mice are kept in standard mouse housing in the animal facility, with temperature, light/dark cycles and humidity maintained as required. Mice also have access to drinking water and clean bedding is provided. Mice are monitored daily for signs of adverse events. Typically, for mice receiving a therapeutic treatment animals will experience mild, transient pain and no lasting harm from administration of substances by injection. Where administration is required for prolonged periods, animals will be surgically implanted with slow-release devices such as a mini-pump. These animals will experience some discomfort after surgery and some mild to moderate pain which will be treated with appropriate pain relief. Animals will undergo changes in diet which are not expected to cause distress but might result in weight loss due to unpalatability. The final procedure will be undertaken under non- recovery anaesthesia where the animals will only be aware of the anaesthetic being administered and may experience mild distress and no pain; they will be culled after this procedure.

To help us to understand the mechanisms involved and the cell types activated, we have also acquired and developed novel genetic mouse strains. Our plan is to breed and use these mice in the procedures described above. Breeding pairs of mice will not be used in any experiments outlined above with the exception of using pregnant female mice at a specific time in their pregnancy to harvest embryos aged between days 14-16 in development . For this pregnant mice, either wildtype or genetically modified (who will not be given the dietary toxin) will be humanely killed (using schedule I approved killing methods) and embryos will be removed and also humanely killed immediately using schedule 1 approved killing methods. We will use the brain tissue from these embryos to grow cells in a dish under sterile conditions.

What are the expected impacts and/or adverse effects for the animals during your project?

Ingestion of the cuprizone toxin for up to 6 weeks is not expected to lead to any adverse events in the mice, however mice may experience some weight loss. Mice will be monitored daily for signs of stress and discomfort. Those mice receiving injections may experience mild transient pain associated with the injection. Some mice will have minor surgery to implant a device under the skin that can release a medicine slowly. They are expected to recover quickly and will be given appropriate analgesia and post-operative care.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severities for:

1. Obtaining embryonic tissue to grow in a dish: 100% sub-threshold
2. Induction of dietary toxin and administration of therapeutic (by way of injection or surgical implantation of a mini-pump): 50% mild, 50% moderate (those mice with minipumps (25%) and/or tamoxifen injection (25%)).
3. Breeding and maintenance of genetically altered mice: 100% mild.

What will happen to animals used in this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Acute cellular responses and injury mechanisms of a single isolated cell type can be studied in reduced systems such as cell culture models. In some instances these culture systems can take advantage of transformed cell lines that can be grown in culture for a long time. However, due to the complex nature of the cells found in the nervous system, these transformed cell lines often have limited capacity to demonstrate the full functions of these cells. Further how these interactions and responses occur within the brain tissue and the involvement of other cell types and matrices within this environment mean that investigations involving cell culture cannot adequately model the complex interactions. Therefore several studies proposed in this project can only be accomplished from animal tissue (primary cell cultures) or appropriate animal models because of the complexity of the interactions between these cells in the nervous system and activated pathways and differing environments within the body. The goal of this research is to understand how the

damage and factors that drive injury do so on cells of the nervous system, and how these cells respond to injury. By providing striking similarities to humans, these animal models and culture conditions have been previously used to demonstrate preclinical in drug efficacy data for approved multiple sclerosis or white matter injury drug treatments.

Which non-animal alternatives did you consider for use in this project?

Alternatives could include utilising commercially available cell lines, such as the mouse derived oli-neu cell line, the HOG human oligodendroglioma cell line, RAW 264.7 mouse macrophages and human ThP-1 cells (macrophages) - however whilst some of these are still animal based, they are immortalized (transformed) lines and may not replicate some of the complex functions of these cell types, but may have use in early in vitro studies (see decision tree)

For this project we also used the 3Rs resource library to search for alternatives using key words 'oligodendrocyte' or 'multiple sclerosis' or 'demyelination' but at this time there are no alternatives available.

Why were they not suitable?

The commercially available lines can be used in some instances, however they are immortalized and with that they are transformed and so how they respond to injury and how they repair may not be the same as using or measuring responses in the same cell type either obtained from primary animal tissue or observed within the animal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We estimate the number of animals to be used in this project based upon good scientific rigor, our pilot studies and prior experience with the use of the models proposed in this application. Using our prior data to model outcomes in an animal model we have previously used, we can perform calculations to estimate animal numbers. Typically using e.g., the cuprizone model, we have found that group sizes of 6 mice provide quality data that demonstrate statistical significance. If we have no prior experience, our goal is to keep animal numbers to a minimum and so we will perform pilot experiments using no more than 2 to 3 mice from each strain. From the data generated in this small cohort numbers can then be expanded to the number required to determine statistical significance (determined by power calculation). Further, we have to take into account the different experimental groups, in most instances, this will be comparing drug intervention to control (vehicle) treated mice, or injury to non- injury mice. In some instances, the number of experimental groups will need to be expanded so to control for experimental conditions, such as with the use of genetically modified mice to model disease and mechanisms of injury. Mice groupings will be per cage groupings and will be randomised

for the different experimental groups. Further scorers/quantitation of outputs will be blinded to the tester to prevent bias. No sham surgeries will be used in this project, for instance, in some experiments mice will have a small osmotic minipump surgically implanted under the skin. The purpose of this is deliver drug for a longer duration and avoid repetitive injections (refinement). In these instances, as drug treatment needs to be compared to vehicle (no drug), all mice will receive a mini-pump implant. For breeding purposes, we use efficient breeding programs, typically keeping to no more than 2 breeder pairs of mice per GMA per 6 month-breeding cycle and have been successful in implementing this approach to maintain colonies and derive experimental mice of desired genotype and numbers for experimental aims. Wherever possible we will attempt to maximise the data output in the animals we use by sharing of tissue for multiple measurements so to reduce animal numbers.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We employed the NC3Rs' experimental design guidance and experimental design assistant (EDA) to plan our experimental design, practical steps and statistical analysis utilising the advice and support for randomisation and blinding, sample size calculations and appropriate statistical analysis methods. We have implemented these practices in our prior research. Further, for instance, in use of the cuprizone model, we follow standard operating procedures for making up the dietary drug and application of it to the mice to reduce inter-variance between experiments and experimental groups.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

By utilising brain cell cultures from embryonic development days 14 to 16 mice, we will significantly reduce and refine our animal use compared to deriving these primary cultures from neonatal mice (mice aged 2-3 days old and typically 5-10 pups per preparation). For neonatal cultures, cells are only minimally expanded in culture and often cannot be cryopreserved thus requiring additional use of neonatal mice for the duration of the experiments. A one timed-pregnant female, with 5-8 embryos, will generate cell cultures (neurospheres) that can be readily expanded and cryopreserved. In our experience we find that one preparation is sufficient to permit multiple and numerous experiments over many years of research. For our immune cell experiments we will only harvest tissue from unused tissue from wildtype mice or surplus mice from other investigators at our institution, thus sharing animals and reducing animal use.

For our experiments with mice at the end of the experiment, we will aim to reduce animal numbers by sharing tissue for multiple outcomes, for instance, a brain can be divided into two equal halves and used in separate analyses. We will also harvest as many tissues as possible at post-mortem. If we don’t need to analyse the tissues immediately, we will freeze them and make them available to other researchers working on similar questions.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The animal model we will use in this study is the cuprizone mouse model of demyelination (loss of myelin). This model is a well-established model of multiple sclerosis (MS) and white matter injury and has many features of the pathology of MS and white matter injury such as damage to specific cell populations within the brain, as well a self-repair modality. Using the 'acute' cuprizone model of demyelination (cuprizone diet for up to 6-weeks rather than the 'chronic' model of cuprizone diet for up to 12 weeks) does not typically have a clinical disease presentation and associated neurological deficit. Typically, with this model mice do not show recognised signs of distress or pain as compared to current alternative mouse models of MS that include 1) immune-mediated demyelination called experimental autoimmune encephalomyelitis. This model causes clinical paralysis in mice and is associated with pain and distress; or 2) direct delivery of a toxin to the central nervous system. This model involves complex surgical procedures directly to the brain or spinal cord in which there is post-surgical pain. The study of nervous system cell functions in a dish can be achieved by harvest and culture of primary embryonic brain and spinal cord tissue.

Why can't you use animals that are less sentient?

For our isolated cell culture (in dish) studies we do use animals, but an immature life stage. For our other studies, in an effort to replicate adult human disease, we use mice that have reached maturity at 8-weeks of age (or 7 weeks of age for reproductive maturity for breeder lines).

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

As part of our ongoing work we strive to refine our procedures with updated practices, recommendations and guidelines. For instance, we have refined our surgical implantation of minipumps by administering additional pain management before the procedure, such as subcutaneous injection of an opioid, prior to surgery, along with local injection at the incision site of additional pain killers to reduce pain and discomfort.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the PREPARE guidelines to inform our practice as well guidance documents provided by the NC3Rs and Laboratory Animal Science Association.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly access the information provided on the NC3Rs website, we will also frequently liaise with our AWERB, NVS and NIO or NACWO to ensure any updates and new practices can be implemented into our project as needed. Further we receive the

NC3Rs newsletter and will arrange to meet with the NC3R regional programme manager.

71. Immunity, Infection and Disorders of the CNS

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

complement system, demyelinating disease, neuromuscular disease, neurodegenerative disease, therapy

Animal types	Life stages
Mice	Neonate, Juvenile, Adult, Aged animal
Rats	Adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overarching goal of the work outlined in this licence is to test the hypothesis that the part of immune system called 'complement' and other related innate immune effectors are important drivers of inflammation and pathology in neurological diseases. This will allow a better understanding of the mechanisms of innate immune involvement in diseases of the CNS and thus give a firmer platform to test new therapeutics and to potentially translate these to human studies. This will be achieved using a structured delivery approach, wherein the role of the immune system, complement and inflammation are examined in three distinct neurological disease areas of interest. Outcome of this project is to improve understanding of innate immune involvement in CNS disorders by focusing on the complement pathway and related immune mechanisms in three specific neurological diseases: Alzheimer’s disease, Myasthenia Gravis and Multiple Sclerosis. This will inform the development of new therapeutics and clinical interventions, ultimately improving treatment strategies for these neurological disorders.

Potential benefits likely to derive from the project, for example how science might be

advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

It is essential to undertake this work to address critical gaps in our understanding of the immune- neurological interface. Its outcomes have the potential to transform our approach to diagnosing, treating, and preventing a wide range of CNS disorders, ultimately improving patient outcomes and public health.

What outputs do you think you will see at the end of this project?

The project is expected to yield several key outputs that contribute to our understanding of the interactions between the immune system (specifically "complement") and neurological disorders. Anticipated outputs include:

1. Research publications: The project will generate research publications in peer-reviewed journals. These publications will detail the methodologies employed, experimental results, and the interpretation of findings. They will contribute to the scientific literature, disseminating new knowledge and insights to the broader research community.
2. Underpinning pathological mechanisms: Here we will investigate mechanisms underlying the relationship between complement system, infection, and CNS disorders. The outputs are expected to include insights into the pathogenesis of specific neurological conditions, potentially uncovering novel pathways or targets for therapeutic interventions.
3. Identification of biomarkers: The project may lead to the identification of novel complement and molecular biomarkers associated with CNS disorders and infections. These biomarkers could have diagnostic, prognostic, or therapeutic implications, providing clinicians with valuable tools for better understanding and managing neurological conditions.
4. Fostering collaborations: The project will allow us to continue working with local collaborators and further allow us to strengthen our external links both in the UK and internationally. These colleagues include a diverse range of researchers, clinicians, and institutions working on CNS diseases, leading to the formation of networks that can further advance research in the field.
5. Generating databases: The accumulation of experimental data, such as genomic, proteomic, and clinical data, will be organised into comprehensive datasets. These datasets will be made publicly available or released upon request, fostering transparency, and facilitating further analyses by other researchers. These databases will also serve as a resource for future investigations in complement and CNS field.
6. Educational purposes: The project will generate educational materials, such as presentations, seminars, or workshops, aimed at disseminating knowledge to the scientific community, healthcare professionals, and the general public. These resources will contribute to raising awareness about the intersection of immunity, infection, and CNS disorders.
7. Improving patients outcome; Based on the research findings, the project may contribute to the development of clinical guidelines and recommendations for the diagnosis, treatment, and management of CNS disorders with an immunological component. These guidelines can inform healthcare practitioners and improve patient care.

8. Technological advancements: The use of cutting-edge technologies in the project may result in technological advancements, such as improved experimental techniques or the development of new tools for studying immune responses in the CNS. These advancements can have broader applications in both basic and clinical research.

Overall, the outputs of the project are expected to contribute significantly to the scientific knowledge base, clinical practice, and the development of interventions for individuals affected by disorders of the CNS with a complement or/and infectious component.

Who or what will benefit from these outputs, and how?

The outputs of the project have the potential to benefit various stakeholders, including:

1. Scientific community: Researchers and scientists working in the fields of immunology, complement, neurology, and infectious diseases will benefit from the new knowledge and insights generated by the project. The research publications, datasets, and biomarkers identified can contribute to advancements in these fields, guiding future investigations and expanding our understanding of the interplay between immunity, complement system, infection, and CNS disorders.
2. Healthcare professionals: Clinicians and healthcare practitioners stand to gain valuable information from the project's outputs. Identification of biomarkers, insights into pathogenesis, and the development of clinical guidelines can enhance diagnostic accuracy, inform treatment decisions, and improve patient management strategies for individuals with CNS disorders influenced by immune responses or infections.
3. Patients and caregivers: Individuals affected by disorders of the CNS, Alzheimer's Disease, and other dementias particularly those with immune-mediated or infection-related conditions, may benefit in the longer term from improved diagnostic tools, personalised treatment approaches, and a better understanding of the underlying mechanisms. This knowledge can lead to more effective and targeted interventions, potentially improving patient outcomes and quality of life.
4. Pharmaceutical and biotechnology industries: The new findings will open avenues for the development of new therapeutic interventions targeting complement, and other immune responses in CNS disorders. This will aid development and testing of novel drugs, vaccines, or immunomodulatory therapies, thereby contributing to the development of more effective treatments.
5. Universities and other educational institutions: Educational resources generated by the project, such as presentations, workshops, and seminars, can be used by educational institutions to enhance teaching and learning in the fields of immunology, neurology, and infectious diseases. This contributes to the training of future generations of scientists, clinicians, and healthcare professionals.
6. Public health authorities: Insights from the project can inform public health strategies related to infectious diseases with neurological implications. Understanding the immune, and complement response to infections in the CNS can aid in the development of preventive measures, vaccination strategies, and preparedness plans for emerging infectious threats that may affect the nervous system.
7. Global community: Given the impact of neurological and neurodegenerative diseases on a global scale, the outputs of this project may aid international efforts to address these public health challenges. Collaborative networks formed as a result of the project may facilitate the exchange of knowledge and resources on a global scale, fostering a collective approach to understanding and managing CNS disorders.
8. Funders and policy makers: Policy makers in healthcare and research funding bodies

may benefit from the project outputs by gaining insights into the importance of supporting research at the intersection of immunity, infection, and CNS disorders. This understanding can inform policy decisions related to funding priorities, research priorities, and public health initiatives.

In summary, the diverse outputs of the project have the potential to benefit a broad range of stakeholders, ultimately contributing to advancements in research, clinical practice, public health, and the well-being of individuals affected by disorders of the CNS.

How will you look to maximise the outputs of this work?

Maximising the outputs of the project involves a strategic and multifaceted approach. Here are several key strategies how to optimise the impact and dissemination of this project outputs:

1. Collaborating with others: We will collaborate with other research institutions, healthcare organisations, and industry partners. By establishing strong partnerships, the project can leverage diverse expertise, resources, and perspectives, leading to more comprehensive and impactful research outcomes.
2. Publishing in open access publications: We will publish our findings in open-access journals to ensure that the scientific community, healthcare professionals, and the general public have unrestricted access to the knowledge generated by the project. This promotes transparency, facilitates knowledge dissemination, and maximises the impact of the research.
3. Attending and presenting at scientific meetings: Presenting findings at national and international conferences and workshops will enable us to engage with the scientific community, share results, and receive valuable feedback. Also, we will host workshops or symposia on project-related topics to facilitate knowledge exchange and collaboration.
4. Informing the Public: We actively and with enthusiasm communicate project findings to the broader community e.g. via Research Institute open events. We will contribute to further activities including public lectures, educational materials, and interactive events aimed at raising awareness about the importance of the research and its potential impact on public health. We will work with media outlets to disseminate project findings to a wider audience. Press releases, interviews, and a presence on social media, will help translate complex scientific concepts into accessible information, reaching a broader segment of the population and increasing public understanding.
5. Generating and sharing datasets: We will make datasets generated by the project available through reputable data repositories (e.g. institutional research portals). This will help promote transparency and allow other researchers to validate findings, conduct additional analyses, and build upon the project's results.
6. Informing policy makers and stakeholders: We will engage with policymakers and advocacy groups to highlight the policy implications of the research. We will communicate the potential impact of the project on public health, and contribute to evidence-based policy discussions related to CNS disorders, immunity, and infection.
7. Planning long-term follow- up studies: We will invest time to plan for long-term follow-up studies to assess the real-world impact of the project's findings. Understanding how research outputs translate into clinical practice and public health interventions enhances the project's overall significance.
8. Generating patent(s) and technology transfer: If applicable, we will explore opportunities for patenting novel technologies or therapeutic targets identified during

the project. We collaborate closely with our Institutional Technology Transfer Office (TTO) to facilitate the translation of research outcomes into tangible applications and products.

By implementing these strategies, we will maximise outputs of the project, ensuring that the knowledge generated has a lasting and meaningful impact on scientific understanding, healthcare practices, and public awareness related to immunity, infection, and disorders of the CNS.

Species and numbers of animals expected to be used

- Mice: 3000
- Rats: 400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

In this project mice and rats will be used. This is a carefully considered choice that aligns with the scientific goals of modelling human disease, ensuring experimental consistency, leveraging established models, and addressing ethical considerations. Many of the mouse strains that we plan to work with have been engineered to be complement deficient (e.g. C1q, C3, C7, CD59), when these are combined (by back crossing) with Alzheimer's Disease model strains such as the widely used APP^{NL-G-F} strain this enables us to study the roles of these complement proteins in neurodegenerative disease in vivo. In terms of our induced models of multiple sclerosis and neuromuscular disease such as myasthenia gravis – experimental autoimmune encephalitis (EAE) and experimental autoimmune myasthenia gravis (EAMG) respectively the use of complement deficient animals alongside wildtype (WT) controls has, and will continue to provide valuable platforms for investigating the intricate relationships between immunity, and disorders of the CNS. Regarding life stages, we will be using animals from within the first week of being born up to 24 months of age. The young animals will be used to understand the role of the immune system in early brain development, what happens to synapses when key proteins are missing and what this implies for adult brain health. Adult animals will be used to study immune involvement in neurological disease and to test potential therapeutics targeted at immune proteins and processes. This latter part of the project clearly needs to be carried out in adult animals and in respect to our work on Alzheimer's Disease it will be critical to study animals at various ages up to 24 months of age.

Typically, what will be done to an animal used in your project?

The main focus of this project is neurodegenerative disease, in particular Alzheimer's Disease. Typically, animals will be allowed to age to planned timepoints (e.g., 3, 6, 9, 12 months) and then killed by a humane method for tissue harvest and analysis. During the study animals may be subjected one or more times to behavioural testing in order to assess the progress of the disease and its impact on cognition. The most commonly used tests we employ are non-stressful and include those such as burrowing and nesting and

novel object recognition.

When we need to test the potential of anti-complement therapeutics in Alzheimer's Disease, we will typically administer these agents to animals via regular injections to test in vivo responses. Blood samples may be collected at various points for analysis of the efficacy of the therapeutic. At the predetermined endpoint, animals will be killed humanely by a schedule one method.

What are the expected impacts and/or adverse effects for the animals during your project?

The expected adverse effects and impact on the animals used during this project will vary depending on the specific procedures involved. Therefore, these are dealt with separately below:

Induction and experimental intervention in models of demyelinating disease (such as human multiple sclerosis). In these models, all animals are expected to develop increasing problems in walking which would lead to eventual paralysis, these affects are accompanied by weight loss over the course of the disease.

The animals are monitored regularly and the impact on their gait scored on a scale of 1 - 4, these are the scientific/clinical readouts and key data outputs for the model. They are indicative that the disease model is working as it is expected and also serve a critical role in our judgement of where each animal is in relation to its humane endpoint.

Induction and experimental intervention in models of neuromuscular disease (such as human myasthenia gravis). Experimental Autoimmune Myasthenia Gravis results in marked muscle weakness and eventual paralysis with associated weight loss, these are the scientific/clinical readouts for these models. As with models of demyelinating disease above, animals are monitored regularly and the progression of the disease assessed by scoring its impact on their paw grip and movement scored on a scale of 1 – 4. This yields both scientifically important data and a way in which to judge approach of humane endpoints.

Induction and experimental intervention in neurodegenerative disease. The predominant part of this work is straightforward and simply involves taking genetically altered (GA) model mice which carry human transgenes important in the development of Alzheimer's Disease and allowing to age to certain experimentally important times. At that point the animals will be humanely killed by a schedule one method for tissue harvest. These genetically altered animals show little or no impact or adverse effects while ageing. Some impact on their cognition can be measured in data from behavioural tests of memory, however these effects have no appreciable impact on the everyday life of the mice.

For some experiments we will assess the impact of common infections, such those induced by lipopolysaccharides (LPS) injections on the progression of Alzheimer's disease (AD). In this work mice will be injected with LPS which cause rigors lasting for up to 24 hours post injections. Animals typically then recover and can be aged as with other model mice.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

For mice and rats, used in models of either demyelinating or neuromuscular disease we expect the severity to be no more than moderate. Where therapeutics are being tested (the typical use of this project) if they prove effective, we might expect that moderate severity only be reached in control animals not receiving treatment.
For mice and rats used in studies of neurodegeneration the expected severity is moderate. However, for the majority (approximately 70%) of the animals on this project we expect the severity experienced to be mild.

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animals models provide complex and integrated in vivo systems that closely resemble human physiology. Studying the immune response, infections, and disorders of the CNS in living organisms allows us to explore intricate interactions and responses that cannot be fully recapitulated in simpler in vitro models. The animal models that we plan to use, (mice and rats), share genetic, physiological, and immunological similarities with humans. Research conducted in these models has the potential for translational relevance, allowing insights gained from the study to be more directly applicable to human health and disease. Use of animals will allow us to identify the best route of administration and the effects of therapeutic interventions, on the entire organism. This is crucial for understanding systemic responses, and critical for our work where we are seeking to delineate interactions between the complement system and the CNS. The animal models we will use allow for the assessment of motor response together with behavioural and cognitive outcomes (e.g. modelling dementia), thus providing insights into the immune involvement in CNS disorders.

Thus, the use of animals in the project is integral to achieving our aims of understanding the intricate relationships between complement, the immune system, infections, and disorders of the CNS. Animal models provide a unique and indispensable platform for conducting experiments that are critical for advancing scientific knowledge and potentially translating findings to improve human health outcomes.

Which non-animal alternatives did you consider for use in this project?

Despite the limitations inherent with in vitro and ex-vivo work flows we shall employ them wherever possible and to the fullest extent to ensure that animal usage is kept to a minimum. Prior to use of any model, we will first employ in vitro experimentation to test hypotheses, supported by experiments using ex vivo tissues and cells. Final testing in

animal models will proceed only in cases where lines of investigation have been validated by these other means and hence justify the use of specific models to prove novel insights into mechanisms of disease or to test new therapeutic agents which have been shown to be effective in vitro (and ex vivo).

Thus, several non-animal alternatives including in vitro cell cultures, human derived cell lines, organoids, organ on chip, ex-vivo tissue cultures, post-mortem human tissue, and computing modelling were considered and will be employed.

1. In vitro cell cultures: in vitro cell cultures provide a controlled environment to study certain aspects of immune responses and cellular interactions. While these cultures offer a valuable tool for preliminary screening of drug candidates and investigating specific cellular mechanisms, they lack the complexity of whole organisms.

2. Human-derived cell lines: The use of human-derived cell lines, especially those relevant to the immune system and neural tissues, allow to examine responses in a more human-specific context.

3. Human cell lines can provide insights into molecular and cellular processes without directly involving animals, however they are not representing the complex immune interactions.

4. Organ-on-chip models: Organ-on-chip technology involves the development of microfluidic devices that mimic the structure and function of organs. These models offer the advantage of simulating tissue interactions and responses in a more controlled and human-relevant setting, but they are not a direct substitute for studying the entire organism and come at high price.

5. Computational modelling: We utilise computational models, such as bioinformatics and systems biology approaches to predict and simulate immune responses, identify potential drug targets, and analyse complex interactions within the CNS. These models contribute to a systems-level understanding of the processes under investigation. However, a life system is required to confirm the modelling predictions.

6. Ex vivo tissue cultures: When possible, we are using ex vivo tissue cultures involve isolating and maintaining tissues from animal models without the need for the entire organism. This approach allows researchers to study specific tissue responses while minimising the use of live animals.

7. Post-mortem human tissues: When available, we outsource from bio-banks post-mortem human tissues provide an opportunity to study the effects of infections and CNS disorders in a more clinically relevant context. This approach allows for the examination of human tissues without direct experimentation on living animals. However, the post-mortem samples are not representative of life organism required for studying drugs effects.

The above-mentioned alternatives offer valuable insights, but they not fully replicate the complexity of in vivo systems. The decision to use animals in the project was made based on the necessity to study the integrated responses within a living organism, considering the multifaceted interactions between the immune system, infections, and disorders of the CNS.

Why were they not suitable?

While non-animal alternatives are vital to the overall program of work, the complexity and integrative nature of the project's goals necessitate the use of animals. Animals provide a more holistic and clinically relevant model for studying immunity, infections, and disorders of the CNS, ensuring a comprehensive understanding of the dynamic interactions within living organisms (including the influence of factors such as circulation, metabolism, and interactions between different tissues and organs). Post-mortem human tissues are very limited in availability, and the range of experimental manipulations that can be performed on such tissues may be constrained. Additionally, studying the progression of infections or responses to treatments over time is not practicable using in vitro approaches alone. Computer modelling methods are also extremely powerful tools but data generated needs to be validated in live animal. Thus, the only way to further our understanding and test novel therapeutics targeting these complex human disease processes is through an integrated approach employed specific and appropriate animal models of disease.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have been conducting the testing of anti-complement agents in CNS diseases for over two decades now. We estimate the number of animals required based on our previous experience. When planning new experiments that significantly deviate from our past research, we extensively review existing literature and a comparable study by other researchers, ensuring reasonable estimations for our experiments. Additionally, we employ power calculations, e.g., using G*Power software or other statistical analysis of data generated from pilot experiments, to determine the overall numbers mentioned above. Considering current funding, available research group staff, and future plans for securing additional grants over the next five years, we believe these estimates represent a fair assessment of the animal numbers necessary to achieve our research goals in the next five years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The estimated numbers are firmly based on previous studies within the group employing similar research objectives, methodologies, and expected outcomes. The estimated numbers are firmly based on previous studies within the group employing similar research objectives, methodologies, and expected outcomes. For the planning of new experiments, we have referred to our published works, which provide detailed accounts of the experimental designs and outcomes, thus ensuring that our estimates are accurate and well-supported.

For the planning of new experiments, where they differ markedly from our previous experiences, we have extensively reviewed the literature to gain insights into the typical number of animals used in comparable experiments by other researchers, ensuring reasonable estimates for our studies. Taken together with statistical testing of data obtained from pilot experiments we have arrived at the overall numbers given above. We believe these to be a fair estimate of the numbers of animals required to fulfil our research aims given current funding, research group staff available and planning for acquisition of further grants over the next five years.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In addition to implementing a robust experimental design, we have built in several measures to optimise the number of animals used in the project, aligning with ethical considerations and the principles of the 3Rs.

Wherever possible we will carry out pilot studies to assess the feasibility of planned experimental procedures, refine protocols, and identify potential variability in responses. Information from pilot studies is crucial in making informed estimates about the required sample size to achieve statistically and scientifically meaningful results.

The estimated numbers of animals were optimised to strike a balance between obtaining scientifically valid results whilst minimising the overall use of animals.

Continuous refinement of our experimental techniques will be a priority to enhance the precision and reliability of data obtained from each animal. Ensuring that procedures are reviewed, critiqued, and optimised minimises the need for additional animals to account for potential variations.

Encouraging collaboration and data sharing with other research groups will be pursued to avoid unnecessary duplication of experiments. This collaborative approach allows for the pooling of data, potentially reducing the overall number of animals needed across different studies with similar objectives.

Wherever applicable, in vitro models will be utilised to address specific research questions. In vitro studies can provide essential data in their own right and link with our research aims here to give us valuable preliminary data helping to prioritise the most relevant experiments to be conducted in vivo thus contributing to the reduction in the number of animals required.

Incorporating longitudinal study designs will enable the collection of multiple data points from the same animals over time. This approach increases the information obtained from each individual subject and minimises the need for larger cohorts.

Statistical considerations, including power analyses and sample size calculations, will be rigorously applied to ensure that the number of animals selected is sufficient to detect meaningful effects. This approach avoids unnecessary surplus in sample sizes while maintaining the ability to draw scientifically valid conclusions.

By integrating these measures into our research framework, we will aim to ensure that the number of animals used will be well balanced with the scientific objectives with ethical

considerations and the commitment to the principles of the 3Rs.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

In this project, we aim to investigate the mechanisms underlying for three groups of human diseases that affect the brain and nervous system and explore potential new therapies based on our findings. These are as follows:

1. Demyelinating diseases such as Multiple Sclerosis (MS) and GuillainBarré syndrome.
2. Diseases of the neuromuscular junction such as Myasthenia Gravis (MG).
3. Dementia and particularly Alzheimer's disease.

Rodents (primarily mice) currently represent the best species available for these studies. This is due to the extensive availability of well-characterised models and a substantial body of published data that supports the design of robust experiments. Our work has utilised the rodent models of MS (EAE, ADEAE) and MG (EAMG) for many years and we are thus very familiar with the particulars of these specific models.

- With this experience and knowledge in hand we will seek to minimise severity through careful monitoring of animals undergoing these protocols. This will ensure that we kill animals before humane endpoints are reached.
- As animals reach clinical scores where movement becomes impaired, we will provide food on the cage floor in the form of wet mash or commercial nutritional jelly to allow access to food for animals which cannot easily reach the food hoppers.
- For disease induction in models of MS we will use the route of administration causing least discomfort (subcutaneous at base of tail), will be carried out with the smallest needle gauge compatible with the adjuvant emulsions (no more than 0.1ml total volume).
- We recognise that these models involve procedures of moderate severity. However, our extensive experience with these procedures enables us to carefully monitor animals and intervene to prevent unnecessary suffering, ensuring that humane endpoints are not reached before a decision is made to humanely kill the animal.
- Regarding our use of murine models of dementia, we have gained considerable expertise over that last ten years with three different dementia models. These models generally cause fewer observable harmful effects on the animals compared to those

used in MS and MG studies. However, we recognise that for some experiments, such those where we are testing the role of viral infection in Alzheimer's Disease there will be a greater burden to the mice. Nevertheless, the experience we have gained helps to ensure that potential welfare problems arising with specific protocols or steps in each model are predictable and that procedures are in place to minimise suffering during the work.

- Throughout the term of the licence, suffering under each regulated procedure will be minimised by expert handling from our cadre of highly experienced research and technical staff, deploying excellent techniques. As on many previous iterations of this licence we will have available and seek as appropriate, support from experienced animal technical staff. Wherever possible and as stated in protocols, animals will be anaesthetised for procedures, (unless doing so would cause more of an impact to the animal); this includes the use of local or light general anaesthesia during blood sampling.

- To reduce the reliance on in vivo studies and ensure the most efficient use of animals, we will integrate a staged approach to in vitro studies:

Stage 1 involves preliminary work using cultured brain cells exposed to purified complement products and other innate effectors to ascertain their effects on isolated cells or cells in mixed cultures. In some cases, brain slices or other organ cultures will be used to test these agents.

Stage 2 focuses on in vitro testing of anti-complement agents to optimise dosing and predict efficacy before administration to animals.

Stage 3 includes ex vivo testing of the ability of agents to localise to activated cells in culture and to sites of inflammation or injury in organ cultures.

This strategy will benefit from significant advancements in tools for in vitro and ex vivo studies, including cell lines, stem cell lines, primary cells, and organ culture systems, ensuring that in vivo experiments are optimally designed to deliver meaningful data from the smallest possible number of animals.

Why can't you use animals that are less sentient?

The use of rodents and especially mice is needed for this for several scientific and practical considerations:

1. Relevance to human physiology: Being mammalian, the rodent models of disease that we will employ mimic many of the physiological and immunological characteristics of humans, making them suitable models for studying human diseases of the CNS. AD, a common neurodegenerative disorder, primarily manifests in the aging population. Use of AD mouse models will allow us to study the disease at stages that closely parallel the progressive nature of the human condition. Alongside studying the detailed molecular changes which occur during the disease we will also be investigating cognitive impairment in live animals at different ages. Therefore, using mice of different ages helps capture the age-related aspects of the disease.

2. Immune system: Mice have immune systems which bear close similarities to our own, a

crucial factor when investigating and testing anti-complement drugs for use as therapeutic agents in human disease.

3. CNS: As with the immune system mice and rats have brain tissue which is very similar in structure and cellular content to our own. The CNS undergoes maturation processes, and adult mice provide a model where the CNS is fully developed. This is particularly important when studying the effects of anti-complement drugs on neuroinflammation, synaptic function, and cognitive measures associated with the studied diseases.

4. Availability of established models: Many of the rodent models we wish to use are very well established, and their use will allow us to leverage existing knowledge and validated experimental systems. This streamlines the research process, aids in the consistency and reproducibility of experimental outcomes and through this contributes to a better, more refined experience for the rodents being used during the term of this licence to understand the role of the immune system in CNS disease.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Potential refinements include increased monitoring, post-operative care, pain management, and training of animals. To minimise welfare costs and harms for the animals involved in the project, a comprehensive strategy focusing on refinement of procedures will be implemented throughout the research process:

1. Conducting preliminary pilot studies: Conducting pilot studies will allow us for the refinement of experimental procedures before the full-scale research begins. These preliminary investigations help identify potential stressors, refine techniques, and optimise protocols (e.g. drug dosing) to minimise any adverse effects on the animals.

2. Behavioural observations: Frequent monitoring will be conducted to promptly identify signs of distress or discomfort. This will allow for real-time adjustments to procedures and ensures that any unexpected adverse effects are addressed promptly.

3. Anaesthesia: As per the standard conditions, we will employ anaesthesia wherever possible, to minimise pain and discomfort associated with experimental procedures. Continual reassessment and adjustment of pain management strategies will be undertaken to ensure optimal welfare throughout the project.

4. Minimising of invasive procedures: Where possible, non-invasive, or minimally invasive techniques will be prioritised to reduce the impact on animal welfare. This includes the careful consideration of alternative methods that minimise the need for surgeries or invasive interventions.

5. Appropriate housing and environmental enrichment: We will provide an enriched environment within housing facilities which promotes the wellbeing of animals and mitigate the potential adverse effects of laboratory housing. Adequate space, proper temperature and humidity control, and comfortable bedding will be employed to contribute to a positive welfare environment.

6. Regular health monitoring: We will routinely perform health checks and monitoring parameters such as body weight, food and water consumption, and clinical signs will

allow for the early detection of any health issues. We will liaise with the local designated animal welfare specialists and employ veterinary intervention when needed to minimise welfare costs associated with illness.

7. Training and habituation: Animals will be acclimated and habituated to experimental procedures through positive reinforcement and training. This approach will help reduce stress associated with handling, injections, or other interventions, contributing to the overall refinement of procedures.

8. Transparency and reporting: Transparent reporting of procedures and outcomes, including any unexpected adverse events, will be prioritised. This facilitates ongoing refinement by providing valuable information for continuous improvement and the sharing of best practices within the scientific community.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

To ensure that experiments are conducted in the most refined way with a focus on animal welfare, we will adhere to and draw guidance from reputable published best practice resources listed below.

1. NC3Rs guidelines: provides a wealth of resources and guidelines for researchers to enhance the welfare of animals used in experiments. The NC3Rs guidelines offer practical insights and recommendations for refining procedures to minimize harm and stress.

2. The Animals (Scientific Procedures) Act 1986 (ASPA), overseen by the Home Office.

3. Guide for the are and use of laboratory animals (8th Edition): provides comprehensive guidance on the ethical and humane treatment of laboratory animals. It offers insights into the proper housing, care, and use of animals in research settings, emphasising refinement strategies to enhance welfare.

4. ARRIVE (Animal Research Reporting of In Vivo Experiments) Guidelines: provide recommendations for the transparent and comprehensive reporting of animal experiments. Adhering to ARRIVE guidelines ensures that experimental details, including refinement measures, are clearly documented, contributing to the dissemination of best practices.

5. Laboratory Animal Science Association (LASA) Guidelines: LASA provides guidelines on various aspects of laboratory animal science, including the care and use of animals in research. These guidelines offer practical recommendations for refining procedures and enhancing the welfare of laboratory animals.

6. Journal of Neuroscience Methods and Other Relevant Journals: Publications in specialised journals, such as the Journal of Neuroscience Methods, often include refined methodologies and best practices for conducting experiments in neuroscience research. Regularly reviewing articles in relevant journals will help the project stay informed about emerging refinement strategies. By incorporating insights from these

best practice resources, the project aims to uphold the highest standards of animal welfare and ensure that experimental procedures are conducted with utmost refinement. Regular updates and reviews of these guidelines will be integral to maintaining a dynamic and responsive approach to the evolving landscape of animal research ethics and best practices.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

To stay informed about advances in the 3Rs and effectively implement these advances throughout the project, a proactive and dynamic approach will be employed:

1. Continuous literature review: We will conduct a regular and systematic reviews of scientific literature, journals, and publications focused on advancements in 3Rs principles. Staying abreast of the latest research findings and methodologies will ensure that the project benefits from the most recent innovations in animal welfare and experimental design. We will implement technological innovations and advancements that contribute to the refinement and reduction of animal use. This may include the adoption of alternative models, in vitro systems, and advanced computational approaches that align with the principles of the 3Rs.

2. Engagement with 3Rs organisations: Collaboration and engagement with organisations dedicated to promoting the 3Rs, such as the NC3R and other relevant institutions, is well established. During my term as an ECR-3R representative, which ran from September 2021 to January 2024, I participated in talks in a number of ECR groups to spread the word about the necessity of using animals in research.

3. Networking with experts: We have an established connections with experts within the field who conduct animal research. We will maintain communication with experts in the field of animal research ethics, welfare, and the 3Rs will be a priority. Regular consultations with professionals who specialise in animal welfare science and ethical considerations will offer valuable insights and guidance. We will leverage resources provided by organisations and initiatives focusing on the 3Rs. Tools, guidelines, and databases offered by institutions like the NC3R will be regularly consulted to ensure the implementation of the latest advancements in experimental design, animal welfare, and reduction strategies.

4. Collaborating with ethical review boards: We will regularly interact with the local ethical review body (e.g. AWERB) and committees overseeing animal research within the university which will facilitate the exchange of information on evolving ethical standards and expectations. Collaboration with these entities ensures that the project remains aligned with the latest ethical guidelines and practices.

72. Regulation of immunity and haematopoiesis

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Immunology, Lymphocytes, Innate lymphoid cells, Disease, Inflammation

Animal types	Life stages
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim is to identify novel pathways that regulate immunity and haematopoiesis during homeostasis and disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The immune system has evolved to protect the host and fight disease. It works throughout the body, interacting with the multitude of organs and tissues, and the nervous system, to maintain immune homeostasis and tissue repair, and defend from infection and damage. However, there are occasions when the host immune system fails to combat the attack.

For example, the World Health Organisation (WHO) estimates that virally and bacterially-induced Pneumonia and diarrhoea combined lead to 29% of all child deaths globally; that over 880 million children require treatment for helminth parasites; and that in 2017 there were 219 million cases of malaria (a protozoal parasite) worldwide with 435,000 deaths. By

understanding the immunobiology of infection we aim to discover new opportunities for better prevention and treatment of disease, to reduce human suffering. For instance, one of the greatest achievements of the 20th Century was the almost total eradication of poliomyelitis following the development of the polio vaccine (99% reduction from 1988 to 2010). We also witnessed the importance of immunology research with the successful development of vaccines to combat COVID-19 at the start of the 21st century. However, for many diseases we still have no working vaccine.

The immune system may also over-react leading to auto-immunity such as inflammatory bowel disease, or may be inappropriately elicited e.g. when pollen or house dust mites cause allergy and asthma. In many of these inflammatory diseases the immune system becomes dysregulated and anti- inflammatory responses that should protect from excessive collateral damage become misdirected.
Asthma is a common chronic inflammatory disorder that is characterised by inflammation and hyperreactivity of the airways, and afflicts approximately 235 million people worldwide including 1 in 5 children in the UK. Significantly, 5 – 20% of sufferers develop severe asthma that is poorly controlled even by newly developed treatments (arising from immunology research). Furthermore, there is growing awareness that acute asthma exacerbations are often associated with viral infection. WHO also predicts that by 2030 chronic obstructive pulmonary disease (COPD), for which there is no effective treatment, will become the third leading cause of death globally. It is critical that we understand the fundamental regulation of the immune system so that we can continue to develop new therapies to relieve human suffering.

Notably, in some circumstances the cellular and molecular peacekeepers of the immune system can be silenced and fail to destroy abnormalities like cancer. It has been estimated that in 2018 ~9.6 million people died from cancer (WHO). However, recent studies of the immune system, relying heavily on fundamental experiments using mice, have led to the development of new therapeutics that prevent immune suppression and enhance immune-mediated killing of cancer cells. Whilst this is a significant breakthrough in cancer treatment, and yet another validation of the importance of studying the immune system, not all cancers can be treated successfully with these new drugs, highlighting the importance of continued work to discover new targets.

Interestingly, the maintenance of immune balance impacts on tissue homeostasis. For example, a lean healthy body is associated with the presence of a type-2 immune microenvironment, whilst obesity is associated with type-1 immune reactions. The incidence of obesity is increasing (1 in 4 adults, and 1 in 5 children in UK are now considered obese - NHS), with significant effects on susceptibility to other diseases including cancer and asthma. Once again, it is critical that we investigate the contribution of the immune system to the deleterious effects of the over-consumption of food.

In summary, this project will investigate the fundamental regulation of immune responses to identify new targets for therapeutic intervention.

What outputs do you think you will see at the end of this project?

The immune system is critical for the defence of the body from infection and cancers, but it can also misfire and start to attack the body (autoimmunity). Infectious disease is the biggest killer of mankind world-wide. Viruses alone kill more than twice as many people each year as cancer. Asthma is a common chronic inflammatory disorder that is

characterised by inflammation and hyperreactivity of the airways, and afflicts approximately 235 million people worldwide. Although many asthma patients respond to corticosteroid medication, such treatment can be associated with negative side-affects. Significantly, 5 – 20% of asthmatics develop severe asthma that is not controlled by standard treatments, and they account for a significant proportion of the morbidity, mortality and cost of the disease.

There is therefore a desperate need to understand how we can selectively arm the immune response to combat pathogens and cancers, or pacify inappropriate immune reactions that harm the host. With this knowledge we can work towards better prevention of disease (through vaccination, prophylaxis and good practice), improvement in diagnosis at an early stage to increase the benefit of treatment and/or reduce transmission, and development of new therapeutics. These are long-term goals, but the objectives of this project are to provide some of the necessary information to make this possible.

Consequently, the immediate core benefit of the work will be that we will increase our fundamental understanding of the molecular and cellular pathways that regulate haematopoiesis (the process of making immune cells) and immunity, especially in asthma and allergy, infection and cancer. A better understanding of these mechanisms and specific candidate genes will allow us, and the scientific community, to develop novel approaches to modulate immunity and blood formation to prevent disease.

We have disseminated our research in over 100 manuscripts published over the past 10 years, with 50 manuscripts in the last 5 years. Furthermore, within a similar time-scale we have produced two novel antibodies that have been humanised and licensed to pharmaceutical companies, as a potential treatment of asthma and allergy. One of the antibodies has completed a Phase I clinical trial. These antibodies may have additional application in cancer treatment. We anticipate that where possible our future findings will be commercialised through collaboration with industrial partners.

Who or what will benefit from these outputs, and how?

The important long-term benefit of our research is to contribute to the development of treatments to both prevent and treat immune and haematopoietic diseases. We have already identified new therapeutic targets and developed drugs that are in preclinical development for the treatment of asthma and allergy and continuing with these studies will enable us to support work to take these drugs forward for clinical trials. In addition, we will continue to pursue the identification of new treatment strategies, for example by using disease models to identify novel gene candidates that can be targeted therapeutically. Indeed, our recent experiments have demonstrated that antibody- treatment of mice with the antibody mentioned above can reduce tumours and double healthy life- expectancy in a mouse model. This pathway appears to be conserved in human samples that we analysed and is associated with reduced disease-free survival. The antibody is cross-reactive with human and has been humanised, and so may be useful therapeutically. In the longer-term we expect our findings to inform and guide new therapies in a variety of human diseases including asthma, allergy, autoimmunity, obesity and cancer.

The generation of transgenic animals within this programme will also be valuable to other scientists aiming to develop therapeutics to these and related diseases by facilitating more rapid progress in their investigations. Also, through publications, presentations and the use of data repositories our work will continue to influence other researchers.

How will you look to maximise the outputs of this work?

It is important that our work is made available to the wider community once we are satisfied that it is robust and reproducible.

We will continue to publish our findings in peer-reviewed journals making use of open access, preprints and journals that accept negative findings or replication studies.

We will collaborate with colleagues in Universities, Medical Centres, other Institutes and the commercial/ biotechnology sector to promote awareness and influence the research direction of others.

We will deposit datasets and analytical tools in repositories and promote data accessibly through careful annotation, metadata and data visualisation applications.

We will share our research tools and know-how freely.

We will protect intellectual property and patent findings when appropriate.

Species and numbers of animals expected to be used

- Mice: 110,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The mouse immune system is extraordinarily similar to the human. While there are differences, these are far outweighed by the similarities; the fundamental appreciation that the cells and genes that regulate immunity in the mouse overlaps substantially with humans has brought fundamental health benefits in a global scale e.g., vaccination, skin grafts, therapeutic monoclonal antibodies, cell therapies. Remarkable discoveries such as regulatory T cells and subsets of innate lymphocytes were first made in mice and then found in humans. B and T cell receptors for antigen are formed by similar molecular processes and their repertoires selected and maintained by similar mechanisms. Similarly, blood cell development is highly conserved between mouse and human.

All our in vivo experiments are performed in mice and many of our other experiments in vitro/ex vivo require cells and tissue from donor mice. Their immune and haematopoietic systems closely resemble those of humans and many models of human disease have been developed in them. The mouse also benefits from well-established and robust technologies for transgenic and gene-targeted genetic alteration. The ability to delete individual genes in mice - in specific cells, at specific times - enables studies that are not possible in humans. The ability to perform infections or study the growth of defined tumours and take tissue samples for research, or examine the effects of one body system (nervous) on another (immune) is impossible or impractical in humans.

Typically, what will be done to an animal used in your project?

To produce gene manipulated mice females will be injected with hormones to increase the production of embryos (superovulation) after which they will be killed to recover embryos. To establish new mouse strains a small number of female mice will receive embryos via surgical or non-surgical procedures. A small number of male mice will be vasectomised to produce pseudo-pregnant embryo recipients, these males will be kept until twelve months of age.

We will breed and maintain genetically altered (GA) mouse strains for experimentation. The maintenance until a maximum of 15 months of age will cover the mice used in this study where no or rare (<10%) mild effects are anticipated. The majority of mice of both sexes will be studied either after killing by a humane method to address questions on immune cell/tissue homeostasis, or under the experimental protocols below to study immune cell and tissue responses. We will recover post-mortem tissues and purify and/or grow cells for use in the studies below.

One important experimental approach we use, common to several of the following protocols is inducible gene deletion. Tissue specific and temporal gene regulation in cells can be achieved using a number of genetic alterations. These studies will involve a minority (~15%) of all procedures.

Most of our experiments in mice will mimic, in an experimentally controlled fashion, the various scenarios the immune system has to deal with (e.g. immune challenge (autoimmune disease (IBD), obesity, asthma and inflammation), infection, cancer). Most experiments are completed within 2 to 4 weeks. Mice will be administered substances (e.g. immune cells, cytokines, antibodies), by a variety of routes, choosing the most suitable and least invasive route possible e.g. most commonly by injection into the peritoneal cavity, in the water or food, via inhalation through the nose, or by injection into the blood.

When we use replicative infectious agents we will, where possible, use attenuated bacterial or viral stains, live vaccines, or expose to doses of infectious agent from which we expect the animals to recover after experiencing moderate severity. This may result in some discomfort similar in duration and severity similar to that of a vaccination or infection, but is rarely found to lead to severe outcomes. In some experiments we may activate, or deactivate, nerve cells to determine if these changes improve or impair the immune response to infections.

Inoculation with tumours or carcinogens will be used to study cancer. These mice will be treated with substances or cells to identify treatments that positively or negatively impact cancer development. In some experiments we may activate, or deactivate, nerve cells to determine if these changes improve or impair the immune response to cancer.

Some mice may undergo surgery (e.g. to enable injection of cells into the kidney capsule) to study immune cell development. Surgery may also be performed to investigate how the nervous system interacts with the immune system.

Inflammatory diseases such as inflammatory bowel disease (IBD) and asthma will be modelled and will be treated with substances or cells to identify treatments that positively

or negatively impact the development of inflammation and disease. In some experiments we may activate, or deactivate, nerve cells to determine if these changes improve or impair the inflammatory immune response.

As an example of an experiment to study asthma-like responses genetically manipulated mice may have the allergen, ragweed pollen extract, administered through the nose for five to ten continuous days. During or after antibodies or cytokines may be administered by three doses into the peritoneum. Two blood samples may be taken to monitor potential improvements in asthma symptoms. The lung function of mice may then be assessed by plethysmography (a procedure ease of measuring breathing, performed under terminal anaesthetic). In some experiments we may activate, or deactivate, nerve cells to determine if these changes improve or impair the immune response to agents such as allergens.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of mice are expected to experience only mild, if any, signs of discomfort and are regularly monitored during studies.

However, some animals will lose weight in response to immune challenge, infection or inflammation. If animals lose more than 20% of their body weight they will be killed as soon as they are identified.

Cancer studies involve the development of tumours. For example, the onset of colorectal cancer can lead to anaemia and weight loss. Such animals are observed at least twice daily and will be killed humanely to avoid suffering should they show signs of ill-health (e.g. weight loss, deterioration in body condition).

Some of the substances used to alter neurological function can have short-lived side-effects including hypo-mobility, lethargy/decreased responsiveness and weight loss. If animals develop these or other signs and fail to improve, they will, depending on severity, be killed humanely, or a programme of enhanced monitoring and care will be instituted until the animal fully recovers. In addition, more refined transgenic approaches are being investigated with the aim to reduce administration of harmful drugs.

In some cases, we will alter neuronal function in mice that will go on to develop tumours. If this requires drug treatment, the mice will be allowed to recover before tumour development is initiated (though this is not possible in some genetic models). In most instances the alterations to the nervous system will be undertaken to prevent cancer development and improve disease-free survival. Animals are closely monitored and will be killed humanely to avoid suffering should they show signs of ill-health (e.g. weight loss, deterioration in body condition). In the relevant protocols all monitoring measures and end points have been added to limit any harms arising.

A few animals might experience weight loss with intermittent bloody diarrhoea as a symptom of inflammatory bowel disease (IBD). We will ensure that the experimental design will keep the number of mice that experience any form of discomfort as small as possible.

Mice will be given pain relief prior to recovery from anaesthesia and whenever necessary

to alleviate pain as advised by the veterinarian.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Approximately 78% of mice will be subthreshold.

Approximately 12% of mice will reach MILD severity.

Approximately 10% of mice will reach MODERATE severity.

What will happen to animals used in this project?

- Killed
- Kept alive at a licensed establishment for non-regulated purposes or possible reuse
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Although we undertake many experiments using cells in culture, the existing in vitro assays do not accurately mirror the more complex molecular and cellular interactions that regulate immune and haematopoietic cells in the body, leaving no practical alternatives to studies in whole animals. Despite the initiation of studies (by others) to perform computer modelling of the immune network these systems do not currently represent a viable alternative to experimental research. In particular, translational aspects of our research, such as the modulation of the immune system, are only possible in live animals.

Which non-animal alternatives did you consider for use in this project?

We do perform numerous in vitro assays to both define the function of the factor or cells under study, and to refine how we should continue the research so as to optimise the information gained. However, even these assays are better performed with primary cells or tissues ex vivo rather than with cell lines that may have an altered state due to their long-term culture. In this way we can better predict how the molecule will act in vivo.

We continue to investigate the potential for using tissue organoids in which innate immune reactions can be investigated, and specific haematopoiesis programmes monitored and manipulated. However, they do not represent a realistic model of the body and show high degrees of variation between individual organoids.
We undertake human studies. With collaborators we refined an allergen challenge model in which skin blisters can be raised at the site of immunisation to give access to serum and cellular infiltrate.

We also have a collaboration to study primary cells from asthma patients in vitro.

Why were they not suitable?

It is not possible to recreate, in vitro, the multi-cellular organisational structure that comprises the immune system. There is incredible complexity to the immune system e.g. immune cells may be produced in the bone marrow, but need to migrate to the thymus to develop and mature, before migrating again to the site of infection to combat disease, before migrating again to provide a memory of the battle so that they can be recalled rapidly if the same infection is detected (even years later).
This diversity of microenvironments and time-scale cannot be reproduced in a culture dish or isolated organoid.

There are critical ethical issues that surround investigations involving human volunteers. The use of human cell culture and blister analysis offers tremendous insight, but before we attempt to manipulate the human immune system we need significant amounts of preclinical information that help to inform on the likely efficacy and potential side-effects.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?
We have over 30 years of experience in the generation of gene-modified mouse strains and constantly assess production success rates to ensure that we optimise and maintain standards of productivity.

We are also implementing new CRISPR-based protocols aimed at reducing the numbers of mice used to produce gene-modified strains and the complexity of breeding programmes used to produce compound strains with gene-modifications at multiple alleles.

We also have extensive experience in managing complex breeding programmes. This may be to generate conditional knockout mice, requiring the intercrossing with specific Cre-expressing mouse strains (both constitutive and inducible), or to assess combined gene disruption to assess gene redundancy. These programmes require large numbers of animals since the probability of generating the correct genotypes decreases with the numbers of alleles involved. Several of our projects involve developing mouse models with multiple genetic modifications.

We have estimated the number of animals we will use based on our previous studies using these protocols. The numbers of mice required for the generation and rederivations of genetically altered mice are based on extensive experience of staff who regularly perform these protocols.

The use of colony management software and knowledge of the breeding performance of

individual strains has enabled us to predict the numbers of mice of the correct genotype that we will produce from breeding, and the numbers of aged mice that we will need.

The group have attended courses on experimental design and statistical approaches and have sought further statistical advice from colleagues whenever necessary. Experimental animal numbers will be determined using available information, factorial design and power analysis to ensure that minimal numbers are used. Where new protocols with unfamiliar end-points are undertaken statistical advice will be sought to ensure group size and experimental design are sufficient to account for the expected variance. Pilot studies, will be used to test the practicality of experimental design and provide estimates of variability for power analysis to determine future sample sizes. Where appropriate, experiments will exploit a within-sample repeated-measures design to maximise the statistical analysis from fewer animals. We have factored in the need for experiments to be replicated independently.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We reduce the numbers of animals used by paying careful attention to the design and planned analysis of the results and use the NC3R's Experimental Design Assistant to ensure we are considering all relevant aspects of design. We perform our experiments in carefully controlled animal facilities that reduce biological and environmental variation; the use of optimised experimental procedures, including the use of genetically identical strains, blinding and randomisation to reduce technical variation; the multiparameter analysis of individual mice; the adaptation of new technologies to create knockouts or other genetic modifications; improving the sensitivity of techniques to enable measurement to be made on small cell numbers isolated from a single animal. For some experiments we are using in vitro methods to promote cell differentiation to defined states under controlled conditions, thus enabling access to cell numbers that would be unfeasible to obtain from mouse tissues.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

There is an on-going cryopreservation programme in which valuable lines are frozen. This both secures the lines and allows us to send embryos rather than live mice to other researchers who have the technical capability to receive them.

Breeding programmes are optimised to ensure as little over-breeding as possible and keep “surplus” animals to a minimum. Our mouse facility has an extremely efficient pipeline of tissue biopsies and rapid genotyping by a bespoke in-house service, that provides timely results to enable efficient breeding.

Where possible we are combining gene reporter alleles to produce compound reporter mice to reduce the maintenance of individual reporter strains.

In order to reduce the numbers of breeding pairs the mice will be kept as homozygous lines (when appropriate and with occasional back-crossing to maintain line integrity), provided that they do not have a harmful phenotype.

To maximise the information from a single animal, we will aim to provide additional tissues

to appropriate scientists so that they do not have to breed mice specifically for their experiments.

We have introduced the generation of gene-targeted mice from in-bred ES cells. This removes the necessity to back cross lines for several generations.

Once characterised, we have made our animals available to the scientific community thereby removing the need to generate multiple animal lines with the same genotype.

We will prepare frozen stores of biological samples (e.g. tissue cells from infection and immunisation studies; bone-marrow; serum; and tissue sections) so that experiments can be performed on previously gathered tissues rather than using new mice.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse has been selected for this project based on it currently being the "lowest" mammalian species in which these experiments can be performed robustly and reproducibly. Their immune and haematopoietic systems are the best analogue for those of humans and many models of human disease have been developed in them. The mouse also benefits from well-established and robust technologies for transgenic and gene-targeted genetic alteration.

We are using a number of interventions, which potentially can lead to clinical signs that mimic human diseases including rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, viral infection, bacterial infection, cancer, asthma and aging. The vast majority of the procedures that we undertake will be mild with a minority being moderate. In fact, we aim to keep suffering to mild in most cases, or failing that moderate since these are more likely to provide us with meaningful information. However, in a small proportion of cases e.g. parasite, viral and bacterial infections and cancer models, it may be unavoidable that the animals develop moderate clinical signs. In particular, this will be the case when we aim to modulate immune responses testing approaches that are intended to reverse rather than prevent the disease. Batches of virus or bacteria, or toxins/drugs may vary in pathogenicity or toxicity, whilst different strains of mice may vary in their resistance or susceptibility. To reduce the risk of unanticipated suffering we will first low dose groups of two mice, a further two mice may be tested at a higher dose and so on until an appropriate dose level is reached. We use a comprehensive monitoring system to assess the animals throughout experiments. Suffering will be minimised by provision of analgesia where appropriate, provision of diet-gel food on cage floors should animals have difficulty accessing water due to mobility impairment, sub-cutaneous hydration if dehydration is apparent, housing in heat room/on heat pad if temperature drops significantly (e.g.

following anaesthesia).

Why can’t you use animals that are less sentient?

Mice are currently the "lowest" mammalian species in which these experiments can be performed robustly and reproducibly. Their immune and haematopoietic systems closely resemble those of humans and many models of human disease have been developed in them. The mouse also benefits from well-established and robust technologies for transgenic and gene-targeted genetic alteration.
Early-stage mouse embryos are unsuited to these studies as the adaptive immune system and immunological memory is a feature of adult animals. However, we will use embryos to study lymphocyte development where appropriate, including using in vitro thymus organ culture. We do use terminal anaesthesia when studying lung function as a readout of asthma-like disease in the lungs.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We use comprehensive monitoring systems to assess the animals throughout experiments. This will be refined if/when additional methods of health monitoring are identified as useful and robust.
We are always seeking additional measures of disease and the harm it induces and employ both quantitative and qualitative assessments of this. We seek to minimise the number of procedures, eg dosing, required to induce or treat disease. We follow the latest research to determine if new methods, strains or detection systems can be employed to reduce the time of each experiment, the number of mice and the severity of the disease itself (for instance whether a new more sensitive measure can be employed to allow a milder form of the disease to be studied).

We will implement appropriate refinements developed by our animal house staff, who have a long history of innovative practice, including environmental enrichment.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Information from the NC3R website ensures we use the most refined experiments. The ARRIVE (https://arriveguidelines.org) and PREPARE (PREPARE: guidelines for planning animal research and testing. Smith AJ et al) are used when planning experiments to ensure we generate meaningful data. Surgery will be performed to best practice as laid out in the LASA guidelines (http://lasa.co.uk/PDF/LASA_Guiding_Principles_Aseptic_Surgery_2010.2.pdf). When substances are administered or blood samples taken, best practice will be followed as set out in A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes, Diehl et al.. When planning experiments we will refer to Norecopa (https://norecopa.no/) to identify guidelines, search for alternatives and ensure that we are following best practice.

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How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed about advances in the 3Rs during this project through attention to the work and outputs of the NC3Rs including at an institutional level a system of regular updates highlighting new findings and training around the 3Rs; workshops and webinars; invited seminars on the 3Rs; and seminars on Research Integrity - which covers experimental design and data management. We will actively stay updated with our field of research through collaboration, conference attendance and reading the literature which frequently highlights innovations (e.g., Cas9 methods). We will use Home Office advice made available to us through our dedicated Home Office Liaison. Our technicians are required to regularly attend formal courses (IAT) and encouraged to pass on new approaches.

73. Understanding colorectal cancer risk factors and their role in disease progression.

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Colorectal cancer, Risk factors, Detection, Cancer initiation, Cancer progression

Animal types	Life stages
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult, Aged animal
Rats	Juvenile, Adult, Aged animal

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to better understand colorectal (bowel) cancer (CRC), which is one of the most common cancers in the UK, with over 44,000 new cases each year. By studying how different factors such as genetics, age, sex, diet, and environmental influences affect the development and progression of CRC, we hope to identify ways to improve early detection and create personalized treatments for patients.

To achieve this, the project has two main goals:

Investigating CRC risk factors: We aim to understand how different risk factors affect normal cell function, how tumors begin to form, and how they grow and spread. This knowledge will help us identify when and how to intervene to stop cancer progression.

Studying key genes and pathways in CRC: We will explore how specific genes and cellular pathways contribute to the initiation and development of cancer. We will also examine how external factors, such as diet and chemical agents, influence these processes. Chemical agents can either promote cancer, such as carcinogens or prevent cancer, such as nutraceuticals. Understanding these interactions will help in developing agents for early detection, prevention, and treatment.

This project aims to provide actionable insights for improving CRC prevention, diagnosis, and treatment strategies by systematically studying these areas.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Undertaking this work is critical because colorectal cancer (CRC) has a high mortality rate, but early detection dramatically improves survival outcomes. By understanding the risk factors associated with CRC, we can create tailored screening and detection programs to identify those most at risk. This enables earlier intervention and increases the likelihood of successful treatment. Additionally, understanding the molecular pathways involved in CRC provides opportunities to develop targeted therapies and prevention strategies.

Expected Benefits for Objective 1:

This research aims to define novel cancer mechanisms, enhancing our understanding of how tumors form and progress. By uncovering these mechanisms, we can identify potential intervention points to disrupt tumor development and spread. This knowledge lays the groundwork for designing and testing innovative pre-clinical therapies and preventative strategies.

Expected Benefits for Objective 2:

The identification of new cancer-related pathways will significantly advance our understanding of tumor initiation and metastasis. This insight will help improve early detection methods and enable the development of novel approaches to modify tumor formation and progression. By focusing on both preventative and therapeutic opportunities, the work has the potential to deliver actionable solutions, such as tailored treatments and nutraceutical-based interventions, ultimately reducing the burden of CRC and improving patient outcomes.

This project bridges the gap between understanding cancer biology and translating that knowledge into practical applications, aiming for a profound impact on CRC prevention, diagnosis, and treatment.

What outputs do you think you will see at the end of this project?

The main outputs of this project will be disseminated at meetings and in peer reviewed publications describing the results of our work. These will describe how CRC risk genes are promoting tumour initiation and progression. In addition, the results of this work may

lead to additional work developing preventatives and cancer therapeutics that target the mechanisms we identify. We will also communicate our work more broadly to a wider audience via science communication events, traditional media and social media outputs.

Who or what will benefit from these outputs, and how?

CRC has a high death rate, however, if detected early, this rate decreases dramatically. In the short term, the work will benefit the scientific community, who will be able to incorporate and build on our research in their own research programmes, medium term outcomes will be to how our the proposed risk genes work (studied under our previous project license), if/how they exert a sex effect as inidacted by the Shroom2 data, their role in normal tissue function (our data indicates a certain intestinal cell 'tuft cells' are depleted) and will highlight the cellular pathways they regulate as possible drug targets and therefore impact on risk and prognosis. Our current data on tuft cell depletion is intriguing and further understanding of the role of tuft cells in the intestine could improve knowledge of infection, a known role for tuft cells. In the longer term, understanding CRC risk factors can help tailor screening and detection programmes to those most at risk and we can better understand pathways to target therapeutically and indeed intervene with pre-clinical trials of potential anti-cancer therapies/preventions for CRC leading to patient benefit and wider economic benefits. Hence the work will be of direct interest to surgeons, oncologists, clinicians as well as basic scientists.

How will you look to maximise the outputs of this work?

All work carried out during this project will be published in open access, peer reviewed journals and disseminated at meetings and conferences. All data produced during the project, whether indicating success or not, will be made freely available to other research groups to help inform their own research and we will collaborate with other groups to ensure our work is carried out effectively. We will discuss our work at public engagement events open to the wider public and communicate our findings more broadly using social media and traditional media. The work will also be disseminated to policy makers when appropriate.

Species and numbers of animals expected to be used

- Mice: 18,500
- Rats: 100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Colorectal cancer is very complex and involves a variety of different risk factors, both genetic and environmental, and indeed different tumour components e.g. tumour cells, immune cells, supporting cells (stroma) and blood supply. So, it is not possible to mimic all these processes outside of the body. Experiments in animals better recapitulate the effects of genetics and environment on how tumours grow and as such it is important to study it in

this whole whole-body context.

Mice have many advantages as a model system to study gene function in vivo. Humans and mice share physiological, anatomical, pathological and genomic similarities and can develop both induced and spontaneous tumours. Mice are a small size, with relatively short life span and a short reproductive cycle, which helps to generate data efficiently. Hence most of our work focuses on this model, however we also wish to use rats as a model for a specific piece of work and this is primarily due to increased lymph node size - lymph nodes are one of the first organs that CRC metastases to and so we are exploring how changes in size of the nodes can be detected by ultrasound and inform the stage of the cancer, therefore aiding prognosis and clinical intervention .

Another important aspect are the numerous established genetically altered lines already generated and phenotyped in mice, these can be utilised for both comparison and assessment of genetic interactions/risk factors we have identified and the knowledge of these mice should help to refine our own clinical end-points.

Overall mice can be studied for tumour formation in the appropriate microenvironments and indeed utilised for studies of potential preventative and therapeutic agents in pre-clinical settings.

We will primarily use adult rodents for our experiments, as CRC is generally adult onset and the genetic models we utilise can be induced at the adult stage.

Typically, what will be done to an animal used in your project?

Experimental rodents will be genetically modified, either by breeding to introduce an altered gene or by using an inducible gene system to trigger cancer development. Some rodents will be injected with agents induce gene loss or tumor formation and others will be aged naturally to allow spontaneous tumorigenesis. Rodents will then be monitored for signs of tumor development, including regular imaging via colonoscopy, and samples may be collected for analysis. When tumors become symptomatic, the animals will be humanely euthanized.

Additional procedures may involve the administration of carcinogens or altering environmental factors, such as specific diets or exposure to nutraceuticals, and therapeutics, to assess their effects on tumorigenesis. In some cases, surgical removal of sex organs may be performed to study hormone- related impacts. To induce metastasis or monitor tumor progression, appropriate injection and imaging techniques will be used, and procedures will be conducted under anesthesia to minimize discomfort. This approach ensures humane treatment while allowing detailed investigation of the role of specific genes and agents in cancer development and progression.

What are the expected impacts and/or adverse effects for the animals during your project?

The primary adverse effects of this research program are associated with the development of intestinal tumors. As tumor burden increases, rodents may experience anemia, evident through paling of their feet, and progressive weight loss. Rectal bleeding occurs in approximately 10-20% of rodents due to the presence of colonic or rectal tumors. While generally well tolerated by the animals without other overt signs of distress, this symptom

is closely monitored.

Additional potential adverse effects include bowel perforation, sustained diarrhea, and inflammation as a result of tumor induction procedures, which may also lead to weight loss. In some cases, rodents may develop conditions such as obesity or diabetes, in experimental protocols involving altered diets. Any related adverse effects will be carefully monitored to minimize harm.

Close observation is maintained when anemia, bleeding, or other significant symptoms are observed. Rodents will be humanely euthanized if they show signs of moderate discomfort, defined as three or more of the following symptoms: weight loss exceeding 20%, subdued behavior, lack of interaction with cage mates, severe anemia, sustained rectal bleeding with visible blood on fur, or persistent diarrhea. Additionally, any signs of severe distress, such as visible pain or lethargy, will prompt immediate humane euthanasia.

Rodents not reaching humane endpoints will be euthanized at pre-defined time points according to the experimental protocol, at which time tissues will be collected for analysis. Monitoring procedures, including regular assessments and humane culling criteria, are in place to minimize and manage the impact of adverse effects on the animals.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild severity (60%)

Moderate severity (40%)

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Colorectal tumours are very complex and have a variety of different components e.g. intestinal tumour cells, immune cells, supporting cells (stroma) and blood supply and it is not possible to mimic these processes outside of the body. Hence, only whole body approaches recapitulate the micro- environmental effects on tumourigenesis and as such it is necessary to study it in this context. The involvement of the tumour microenvironment and the immune system on cancer cell development and response to therapy is well documented and highlights the need to use rodent models for these studies. Humans and rodents share physiological, anatomical, pathological and genomic similarities and can

develop both induced and spontaneous tumours. Rodents are smaller, with relatively short life span and a short reproductive cycle and this allows us to obtain data efficiently and consistently.

Overall rodents are considered very useful models to study for tumour formation and prevention in the appropriate microenvironments and indeed utilised for studies of potential preventative and therapeutic agents in pre-clinical settings.

Which non-animal alternatives did you consider for use in this project?

We will utilise datasets (expression correlation, publicly available datasets, genetic analysis), in vitro (CRC cell lines) and ex vivo (intestinal organoids) approaches to help us prioritise pathways, genes and genomic locations which affect gene expression.

A number of ex-vivo tumour and tissue organoid culture techniques can be used in parallel to these studies. Intestinal organoids can be utilised for establishing mechanisms, cellular function and testing the effects of environmental factors, hence reducing rodent numbers. Intestinal tumour organoids will effectively induce certain stages of disease in the models, in a targeted and efficient manner. These alternative models complement the use of animals and can be used for initial target identification and mechanistic analyses.

Bioinformatics approaches, for example mining human gene expression datasets, help determine human disease relevance of selected genes and these approaches reduce the number of animals used by focusing their use on addressing the key questions throughout the proposal.

Why were they not suitable?

Ultimately organoid models fall short of fully recapitulating the complex environment found within tumours. In particular, they lack immune, stromal and vasculature involvement and cannot be used to model more complex tumour phenotypes such as tumour initiation and metastasis. For these reasons these models fall short of replacing the need to carry out these experiments in animal models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Our past experience of breeding and experimentation, has allowed us to estimate the number of rodents required to generate significant results.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To reduce the number of animals used we follow a number of steps:

1) Power analyses/calculations will be performed to identify the required rodent numbers needed to generate significant results (URL: eda.nc3rs.org.uk). We perform short-term studies to examine the immediate effect of gene mutation and long-term studies to examine the development of tumours. We have established and validated various outcome measures. For short-term studies, these include: number of stem cells, differentiated cells and proliferating cells present per intestinal/colonic crypt and target gene expression analysis. For long term tumour studies, the primary outcome is tumour free survival, data being compared to controls and previous studies by appropriate statistical methods (Kaplan Meier survival plots, tumour number/survival by Mann Whitney tests).
2) Embryos/sperm are frozen from lines not immediately required for experimental studies. We will minimise surplus by maintaining/freezing stock animals with multiple genetic modifications and extrapolating controls from different cohorts. Thus, fewer animals of the incorrect genotype will be generated.
3) Utilising IVC units minimises environmental influences and we use littermates where possible to reduce sources of variability and the overall rodent numbers required.
4) Tumour initiation and metastasis is a highly complex process that occurs following the acquisition of multiple genetic mutations. The only reliable model that gives rise to liver metastasis, carries 4 separate genetic alleles (VilCreERT2 P53fl/fl KrasG12D NotchICD). Therefore, breeding these complex genotypes increases the animal usage (over 1000 rodents would be required to generate cohorts for analysis). Organoids from these rodents and tumours can be genetically altered and successfully re-transplanted at a high efficiency (~80%) to help with reducing these numbers.

Therefore, the use of orthotopic transplantation rodent models is a viable, disease relevant alternative with a small fraction of the animal usage.

Throughout our experimental design we have utilised information that we have derived from experiments carried out under our current license to inform this process.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

1) Breeding plans are optimised for efficiency
2) Utilising ex-vivo tissue reduces the animal numbers required to achieve our objectives. We have established intestinal organoids, embryonic fibroblasts, skin keratinocytes and skin fibroblast lines, which can be explored for tumour phenotypes, treated with agents and analysed for cellular function.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why

these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Genetically engineered rodent models or cells will be used throughout this project. In addition, orthotopic transplantation of organoids into recipients will be carried out. Procedures will include injection or feeding with inducing agents (eg tamoxifen, doxycyclin), injection with labelling and novel contrast agents (eg BrdU), induction of colitis (DSS) and carcinogenesis (AOM) and treatment or feeding with candidate therapeutic and environmental agents.

Numerous established genetically altered lines are already generated and phenotyped in rodents, these can be utilised for both comparison and assessment of genetic interactions/risk factors we have identified and the knowledge of these rodents should, and have helped, to refine our own clinical end- points.

Where possible inducible systems will be used to specifically target the tissue of interest thereby reducing off-target adverse effects in the animal.

Why can't you use animals that are less sentient?

These models have been chosen because the gene alterations/mutations that they carry are those associated with the human disease allowing us to recapitulate the influence of these genes in human cancer. No other models are currently available to perform this analysis and lower species such as fish or flies do not accurately recapitulate the human disease. Indeed, a number of studies in fish have demonstrated fundamental differences in the mechanisms of Wnt signalling activation following loss of Apc so this model is inappropriate for modelling human colorectal cancer.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Where possible systems will be used to specifically target the tissue of interest thereby reducing off- target adverse effects in the animal. The group will ensure that all animals receive the highest standard of care and appropriate social, environmental and behavioural enrichment such as bedding, tubing and group housing and will be provided. Close monitoring of tumour development will ensure animal suffering is kept to a minimum. We have vast experience of these cancer models and have developed advanced scoring systems to monitor the clinical signs that develop and can therefore ensure that suffering is kept to a minimum. The colorectal tumour models animals will be euthanized when they reach the clinical endpoints outlined in the project. Reaching these clinical endpoints is extremely important to allow disease progression to a stage where is reasonably mimics the human disease and to ensure all animals with different genetic alterations are taken at an equivalent disease stage thus allowing accurate comparisons between them. Additionally work carried out to date under our current license has demonstrated that using these clinical endpoints, allows us to obtain statistically significant results eg tumour production when a risk gene is removed, enabling study of the role of key proteins in colorectal tumour development, and importantly, minimising unnecessary repetition.

Some lines generated have been given extra bedding and mash when young as they have not gained weight in the same way as their littermate controls. This may be an important phenotype but we will continue to refine as appropriate in consultation with the vets.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

For all our studies we will refer to the Guidelines for the Welfare and use of animals in Cancer Research (Workman et al, 2010) and ensure best working practice. We will use all the refinements (1- 10) recommended in this document. We will also refer to PREPARE and ARRIVE guidelines:

https://journals.sagepub.com/doi/10.1177/0023677217724823
https://journals.plos.org/plosbiology/article?id=10.1371/journal.pbio.1000412

and the following resources:

https://www.nc3rs.org.uk/experimental-design

https://acmedsci.ac.uk/policy/uk-policy/animals-in-research

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Via the 3Rs website: https://www.nc3rs.org.uk and communication from animal facility management.

Staff are kept informed of any advances in 3Rs and will implement them. Changes to protocols as a result of these advances will be implemented via license amendments.

74. Understanding disease mechanisms in frontotemporal dementia and motor neuron disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Dementia, Motor neuron disease, Inflammation, Microglia, Vascular

Animal types	Life stages
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To investigate the mechanisms which cause damage to the brain in frontotemporal dementia (FTD) and motor neuron disease (MND), particularly the role of the immune system in disease. We also aim to investigate changes relating to blood vessels in FTD/MND.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Frontotemporal dementia (FTD) is the 2nd most common form of young-onset dementia, typically affecting people in their mid to late 50s. It is a severe, debilitating and progressive disease causing personality changes, irrational behaviours, loss of inhibition, changes in

social behaviour and emotional processing changes. Around 15% of people with FTD also develop motor neuron disease (MND). MND is a devastating disease which causes the nerves which control muscles to die, resulting in progressive muscle weakness, difficulty with speech, and paralysis which spreads throughout the body. MND is almost always fatal within 2-5 years of onset. Crucially, there are no effective treatments currently available for either FTD or MND, which is why research into these diseases is so important.

People with FTD and/or MND show signs of excessive inflammation in the brain. The brain's immune system goes into overdrive, and immune cells like microglia produce inflammatory chemical signals which are thought to damage the brain in other diseases. However, this has been under studied in FTD/MND, and therefore we still don't understand what causes the inflammation or how it affects brain function. An emerging body of evidence strongly suggests that excessive inflammation does contribute to the brain damage which causes symptoms of FTD and MND in people. If this is the case, it may be possible to treat FTD/MND in future using drugs which target the immune system.

We also know that vascular changes in the brain are important in other types of dementia like Alzheimer's diseases, but again this has not been well studied in the context of FTD and MND. Understanding how vascular changes may contribute to FTD and MND disease progression may also lead to the discovery of new treatments.

We will use mouse models of FTD/MND to investigate both immune and vascular contributions to disease. This project will provide novel mechanistic insights which will be invaluable for development of new treatments for FTD and MND, which are urgently needed.

What outputs do you think you will see at the end of this project?

The primary outputs of this project will be greater understanding of the disease mechanisms underlying both FTD and MND, and possible identification of novel therapeutic targets for both diseases. We anticipate publication of several papers in reputable, open-access journals, and presentation of key findings at major national and international conferences throughout the project. A large dataset detailing changes in gene expression in FTD/MND will also be published, which may be used for further analysis by other groups in future to avoid use of additional animals. Finally, the project will generate novel genetically altered animals (GAAs) with altered immune function (details in Protocol 1), which will be useful for a wide range of research application in future (basic immunology research and research into many diseases beyond FTD and MND) and these will be made freely available once published.

There is high potential of findings from this project translating to clinic in future (beyond the duration of the project), as findings will direct future drug discovery research for FTD and MND.

Who or what will benefit from these outputs, and how?

Our own research group and collaborators will benefit from these outputs as they will progress our research interests and direct future our research. Other research groups will benefit from our published papers, datasets and conference presentations as we expect to make major contributions to the field which will inform future research directions.

Our ultimate goal is for FTD/MND patients and their families and carers to benefit from our work in the long-term. We hope outputs from this project will contribute to future drug discovery research in a meaningful way, improving the quality of life of those affected by these devastating diseases.

How will you look to maximise the outputs of this work?

This project is highly collaborative, with experienced colleagues contributing technically and scientifically locally and at other institutions. This will maximise our scientific outputs.

Our findings will be published in reputable open-access journals in line with faculty guidelines and our primary funder's policy (Alzheimer's Society). We will also disseminate findings at conferences and in open dialogue with colleagues internally and externally and we welcome feedback, ideas and new collaborations.

Alzheimer's Society also assigns 2-3 lay volunteers with personal experience of dementia to each grant holder to act as "monitors" for the project. We will engage regularly with our research monitors to ensure our research does not lose track of our long-term goal and stays relevant to the needs of people affected by FTD/MND. These meetings are usually a good reminder of the importance of our work and can be very motivating for early-career colleagues, which further maximises output.

Species and numbers of animals expected to be used

- Mice: 2400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We have chosen to use mice because this is the only species for which all of the required genetically- altered strains exist, and in which the clinical and pathological features of human FTD/MND can be closely mimicked. Mouse is also the most appropriate species for the techniques required, such as behavioural tests (which will be used to measure the severity of cognitive deficits and movement problems) and imaging techniques such as MRI scans.

The project requires mice to be used as neonatal pups and maintained into adulthood. This is because FTD and MND are progressive age-related diseases where symptoms begin in adulthood and worsen over time. We will inject reagents directly into the brain of neonatal pups, to introduce a genetic alteration that mimics a known genetic cause of both FTD and MND in people. This method has been published previously and used by several other research groups successfully. After a single injection, mice develop problems with cognition and movement by three months of age, and these are more severe by six months. We aim to modify immune/vascular function in FTD/MND mice using drugs or genetic alterations, and investigate how these changes impact the severity of deficits, age of onset, or rate of progression. Therefore experiments must be performed in adulthood,

after the onset of deficits. Since the deficits worsen with age, mice will only be maintained as long as necessary for each specific experimental aim.

Typically, what will be done to an animal used in your project?

Genetic alterations (GAs) are required to create mouse models which mimic the clinical and pathological features of human FTD and MND. These alterations will be achieved either by breeding stable GA mouse lines, or by injecting neonatal pups in the brain to induce the genetic change. Some mice will also have an additional GA to alter immune function, or else a drug (e.g. an anti-inflammatory drug) may be delivered via food, drinking water or by injection. The least invasive delivery method possible will always be used.

Animals will be aged (typically no more than six months), and those with FTD/MND-related GAs are expected to develop progressive cognitive deficits and movement difficulties during adulthood. We will measure these deficits using behavioural tests, for example maze tests to measure memory, or timing how long the mouse can balance on a rotating bar to test movement ability. The tests are not invasive. Several tests will be performed to ensure robust data is obtained, and this will usually be repeated two or three times throughout adulthood, for example at 3 and 6 months of age. This is to measure the severity of deficits, the age of onset, and the rate of progression. We aim to investigate whether any of these parameters can be changed by modifying immune function, which would inform future drug discovery research for FTD/MND. For example, could an anti-inflammatory drug administered in middle age delay the onset of FTD/MND symptoms, or could a drug administered after the onset of symptoms slow down their progression and improve quality of life?

A smaller number of animals will also undergo imaging procedures such as MRI scans, which will be done under general anaesthesia. We may also take blood samples (up to a maximum of six occasions per animal, at volumes which will not cause adverse effects or distress) or measure blood pressure (usually weekly) in some mice as required.

All mice will be killed by humane methods once experiments are completed. This usually will be done under terminal anaesthesia, in a way which allows maximum collection of tissues and samples (e.g. blood, brain, spinal cord). This will allow us to perform additional experiments and maximise the data output from each animal.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice with altered immune function (induced by genetic alteration or administration of substances e.g. injection of anti-inflammatory drug) may have increased susceptibility to infection, although mice will be kept in a clean environment, so infection risk remains low. Mice with altered immune function may also exhibit sickness behaviours e.g. changes in food intake/body weight, grooming, nesting/burrowing behaviours. These adverse effects are expected to be mild in severity.

Mice expressing FTD/MND genes are expected to develop progressive cognitive and locomotor deficits reminiscent of human FTD/MND as they age. Based on published models, these may include memory impairments, altered gait and muscle weakness/movement difficulties. We expect these impairments to develop by approximately 3 months of age and continue to worsen with age. These adverse effects

are necessary to investigate disease mechanisms in FTD/MND and are not expected to exceed moderate severity. Humane endpoints for locomotor deficit in particular will be observed throughout the project to ensure animals do not experience excessive distress or health complications, and that deficits do not exceed moderate severity.

The majority of our experimental aims involve understanding changes in brain function that occur early in disease, prior to the onset of cognitive/locomotor deficits, or investigating factors which impact the progression of disease. For example, we will investigate whether drugs which reduce immune function can delay the onset of clinical signs, reduce their severity, or slow their progression. As such, most experimental procedures will be performed on animals in the early stages of the disease. Since adverse effects will worsen over time, animals will not be aged any further than required for the specific aims of an experiment, to ensure adverse effects do not become more severe than is absolutely necessary.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Approximately 75% moderate and 25% mild or subthreshold.

What will happen to animals used in this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to use animals to monitor behavioural phenotypes as one of our key experimental readouts of FTD/MND disease progression and severity. We also require animals to investigate whole organs/systems which cannot adequately be replicated in vitro like investigation of blood-brain barrier (the protective barrier which keeps the brain separate from other organs, and prevents toxins getting into the brain from the blood) integrity and infiltration of immune cells into the brain.

Which non-animal alternatives did you consider for use in this project?

We have an ongoing programme of in vitro work in this field which will continue to be used for any experimental aims which can be achieved in this way. However, the experimental aims of this particular project can only be achieved in vivo.

We also considered using unregulated animals such as C. elegans worms, Drosophila or Zebrafish larvae for this work.

Why were they not suitable?

In vitro methods would not facilitate monitoring of locomotor and cognitive function, or investigation of whole systems such as blood-brain barrier integrity or infiltration of peripheral immune cells into the brain.

We have previously been successful using worms and fish for investigation of motor phenotypes relevant to MND, and there are also published models using flies, although these models are less useful for investigation of cognitive deficits relevant to FTD. There are also conflicts in the published literature between mouse and zebrafish models of FTD/MND, with mouse models more closely mimicking the human disease.

In addition, the immune system has a great deal of inter-species variability, and therefore mammalian models are most suitable for this work. Furthermore, we require the use of in vivo imaging techniques which are not available in lesser species such as flies, worms and fish to study immune and vascular function e.g. PET scans.

Finally, mouse is the only species for which all the relevant GA strains required are available, as this project requires a combination of several different genetic modifications.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have consulted with a biostatistician with experience of in vivo behavioural testing to determine group sizes required for experimental cohorts, and estimated the number of experimental conditions and timepoints we will require to achieve our objectives.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We employed the NC3Rs' experimental design guidance and experimental design assistant (EDA) to plan our experimental design, practical steps and statistical analysis utilising the advice and support for randomisation and blinding, sample size calculations and appropriate statistical analysis methods. We will use the EDA diagram and report outputs to support experimental planning with animal users.

We have chosen models of FTD/MND with the least variability to reduce necessary group sizes for behavioural testing.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For any questions which could be answered using alternative methods such as immortalised cell culture, we will do this instead of using animals.
At the end of experiments, we will harvest as many tissues as possible at post-mortem. If we don't need to analyse the tissues immediately, we will freeze them and make them available to other researchers working on similar questions. We will also share organs used for primary cell culture preparations with colleagues (e.g. thigh bones to isolate immune cells from bone marrow) to reduce the number of mice used overall between multiple groups.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use several different GA models of FTD/MND, either stable lines or GAAs generated by injecting virus-like particles into the brain of pups to trigger expression of FTD/MND-related genes. In order to study FTD/MND, animal models which display clinical signs reminiscent of the human diseases are required. Therefore there is no way to avoid some degree of suffering, primarily through locomotor and cognitive deficits. Animals will be closely observed and humane endpoints will be followed to ensure adverse effects do not exceed moderate severity. Experimental procedures will always be performed at the earliest timepoint possible, to minimise progression of locomotor deficits.

Why can't you use animals that are less sentient?
The immune system has a great deal of inter-species variability, and therefore mammalian models are most suitable for this work. Furthermore, we require the use of in vivo imaging techniques which are not available in lesser species such as flies, worms and fish to study immune and vascular function e.g. PET scans.

Finally, mouse is the only species for which all the relevant GA strains required are available, as this project requires a combination of several different genetic modifications.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will keep up to date on the literature regarding best practices and updated protocols for all procedures. We will continue to use resources such as the NC3Rs EDA and adhere to the PREPARE and ARRIVE guidelines.

The major concern for animal welfare throughout this project is progression of locomotor deficits, which may cause distress and limit an animals' ability to access food and water. Mice developing locomotor defects will be closely monitored by trained technicians/researchers and once severity is considered moderate for a particular animal,

more detailed welfare checks will be performed regularly with clear humane endpoints observed. Floor feeding and/or provision of food and water via mash and gels will be used if required to improve access.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the NC3Rs EDA and PREPARE guidelines to assist with experimental design and study planning, and follow the ARRIVE guidelines when publishing our findings. We will also keep up to date with literature and resources on best practice including those shared by the BSF at our establishment and experienced colleagues and collaborators.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly check information on NC3Rs website and sign up to the NC3Rs newsletter, check internally distributed updates at our establishment, and attend relevant seminars and consult with colleagues and collaborators regularly.

75. Gene Therapies for Neuromuscular Degenerative Diseases

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Gene therapy, Muscle, Heart, Neuromuscular diseases, Pharmacological approaches

Animal types	Life stages
Mice	Neonate, Juvenile, Adult, Aged animal

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall objective of this project is to test pre-clinical gene therapy protocols for inherited neuromuscular and cardiovascular diseases prior to clinical trials.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This project will study several antisense, pharmacological and gene therapy strategies to treat neuromuscular and cardiovascular diseases conditions. This is important because, as yet, there are no curative therapies for disabling and lethal conditions like Duchenne muscular dystrophy (DMD) and other muscle diseases affecting children and adults.

What outputs do you think you will see at the end of this project?

The expected outputs are advanced gene therapy reagents and insights towards development of clinically realistic gene therapy strategies to counteract Duchenne muscular dystrophy and other neuromuscular diseases. The work generated with this project will be published in high impact scientific journals and will be disseminated to the public by means of scientific conferences and workshops and webinars for lay people.

Who or what will benefit from these outputs, and how?

In the short term this research will impact the field of preclinical development for muscular dystrophy. Scientists will primarily benefit from the data obtained with these studies through data presentation on conferences and by means of scientific publications. In the longer term other stakeholders will benefit like companies that will be able to work on the agents generated during this research, clinicians that will be able to provide and test new treatments and most of all patients affected by neuromuscular conditions that will be the end-users of such agents. The most effective and promising agents identified will be IP covered and licenced to companies so that more advanced studies can be performed in larger animals or larger animal models of diseases. This in turn will allow to collect the data necessary to obtain approval from regulatory agencies to perform clinical test in human that, if successful, will pave the way for the commercialization of new treatments.

How will you look to maximise the outputs of this work?

The results obtained from this project will be published in high impact peer reviewed journals and where possible in open access journals that guarantee visibility to the data.

Data will also be presented at local, European and international conferences where gene therapy, antisense and pharmacological approaches for treatment of human diseases are treated, and where unsuccessful approaches can be discussed

The work proposed here will enhance the collaboration with established partners and will create new links with other academics and industry.

The principal Investigator will be responsible for academic dissemination of results, and for working with the College's Press Office to ensure material produced for lay audiences is accurate and comprehensible.

Species and numbers of animals expected to be used

- Mice: Approximately 9,000 mice will be used over the course of this 5-year programme of work.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The mouse is a small mammal, relatively cheap to maintain and presenting a genome that is, for several aspects, similar to the human one. We have been using murine models of

neuromuscular conditions for many years and we and others demonstrated the relevance of some of these models in translating new therapeutic applications in clinic. A number of murine strains will be used for these experiments. Their use is justified by the fact that they either present genetic mutations that make them established and reliable models of the diseases we are studying (Duchenne muscular dystrophy, Oculopharyngeal muscular dystrophy, Facioscapulohumeral muscular dystrophy) or they can become models of such diseases after treatment. They very well model several aspects of the condition in human. Specific genetic or pharmacological approaches designed for their treatment represent a first step towards the development of a cure for human conditions.

The treatments we are developing need to be performed at different life stages, in some cases starting from newborn mice. For the therapeutic approaches, mice are generally treated either when adult or at very young stages (2 weeks onward) to mimic the age of treatment we would perform in human. In some cases the treatment needs to start earlier (in newborn mice) when particular features of the disease (e.g. fibrosis deposition) needs to be prevented as its reversion is not possible with the current strategies. The generation of a new model of disease needs to use newborn mice to generate the phenotype in the whole body.

Typically, what will be done to an animal used in your project?

Animals will be bred in sufficient number to keep the colonies and to provide the animals for experimentation. Mice will be injected using different delivery routes with agents at various dosages.

Agent administration may, in some cases, require surgery. Treatments will be evaluated using a number of outcome measurements based on established physiology assessment. Some of the mice will be used to prepare primary cell cultures to test agents before their use in vivo.

What are the expected impacts and/or adverse effects for the animals during your project?

The mice under study here will undergo different procedures. Regular checks will ensure that any rarely expected adverse effects (i.e., acute distress, extended bleeding at or infection surround injection site, over/under anaesthesia, gastrointestinal irritation, bodyweight loss, cardiac/respiratory failure) are rapidly discovered and managed (i.e., by monitoring continuously until animals fully recover, using analgesic cream to reduce pain or stop bleeding, providing hydrated food chow/mash, using antibiotics, limiting high doses of gene therapy reagents to mice < 6 months). Effective pain relief and anaesthetics will be used rigorously to minimise the severity of the procedures. Animals having prolonged effects or losing >20% of bodyweight will be killed by a Schedule 1 method.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Moderate severity: 35% of animals (mice)

Mild severity: 65% of animals (mice)

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The primary purpose of the research programme is to develop human gene therapies for muscle conditions. A range of gene therapy treatments and pharmacological approaches will be designed, and routes of administration evaluated for effectiveness and safety. Some studies on purity and functionality of reagents can be performed in vitro, but we are not aware of non-in vivo methods that allow evaluation of physiological responses to prototype gene therapeutics in the context of generalised muscular function. The FRAME (https://frame.org.uk/) and NC3Rs (www.nc3rs.org.uk) websites list alternatives to animal experimentation that have been examined without success. Thus, the evaluation of prototype gene therapies in terms of pharmacodynamics and pharmacokinetics, effectiveness of routes of administration, dose-response relationships, and muscular, cardiovascular, neurological and immunological function require whole animal investigations. These animal experiments are essential steps in translating new gene therapies into human clinical trials. We are not aware of an alternative way of directing gene expression in cells that would enable this proposed study to be carried out successfully and in a practical manner, thus we believe their use to be justified.

Which non-animal alternatives did you consider for use in this project?

Preliminary studies were performed in conventional cultured cells (myoblasts and myotube cultures) and cellular models of relevant diseases (cell cultures of immortalized myogenic cells obtained from patients carrying the genetic mutation leading to the disease) prior to translation into animal studies. These studies are crucial to identify and optimize the agent under development and for a relevant preliminary test and are routinely performed in our laboratory. We have also considered the use of 3D culture models and initiated a collaboration with other academics to perform such cultures and test our gene therapy treatments and pharmacological approaches.

Why were they not suitable?

Cellular models are very helpful options for screening gene therapy and pharmacological reagents. However, they are not suitable for evaluating in vivo safety and physiological efficacy of therapies. These need to be assessed in more complex settings where organs and tissue systems are connected by vascularization and innervation which is currently not available with in vitro cellular systems.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated animal number has been predicted based on the animal use in our previous project licences. As the type and the amount of proposed work are not significantly different from what was described in the previous projects, we believe that a similar amount of animals will be necessary for this 5-year study.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Before embarking on regulated procedures, all gene therapy reagents, antisense therapeutics and pharmacological treatments will be analysed in vitro for appropriate design, concentration, activity and purity. In addition, as much as possible, in vitro evaluation of vector configurations (serotypes, promoters, etc) will be made so that only the most effective test therapies are taken forward to animal experimentation. Groups of negative (sham- or placebo-treated) disease models and positive (sham- or placebo-treated) wild-type non-disease strains will be routinely incorporated on the experiment as control animals. The use of such control mice will make reliable the evaluation of pathology correction. The number of animal groups and group sizes will be decided based on previous experience and in keeping with the NC3R's experimental design guidelines, appropriate statistical power analysis and expert advice; typically 8-10 mice/group will be used but the real number will be calculated case by case for each experiment using statistical software (e.g. GPower3).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where appropriate, qualitative pilot studies will be performed in limited numbers of animals to ensure basic functionality (for example to test tropism of new vector configurations). Fully quantitative animal studies on the effectiveness of the therapeutic reagents and administration protocols will be conducted with group sizes predicted from prior knowledge of the scale and variability of measured parameters (histological, biochemical, behavioural, electrophysiological) to ensure adequate statistical power. Furthermore, software for statistical power analyses (e.g. GPower, PASS and similar programs) will be used to estimate the suitable number of mice to be included in the study. Advice on experimental design, cohort group sizes, statistical power calculations and relevant statistical tests will be sought from statisticians. Statistical significance in measured parameters between treatment and control groups will be assessed by standard t-test and ANOVA tests. Careful evaluation of the transgenes to be used will be estimated from in vitro tests prior of injection in vivo. Notably, procedures will be performed in the morning whenever possible to allow regular check over the following hours to make sure the injected animals are observed for possible adverse events and, if relevant, that dams accept and feed the injected pups.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The primary purpose of the research programme is to develop human gene therapies for muscular dystrophy and muscular atrophy. A range of animal models exists for some of these conditions (mouse, rat, rabbit, dog), however, mice are a widely accepted experimental model for pre-clinical and therapeutic studies. The following clinically-relevant mouse models of human disease will be used in these studies:

Mdx Mouse: The mdx mouse is a naturally occurring animal model of human Duchenne muscular dystrophy (DMD). It is generated under the C57BL10 background strain. It has a point mutation in exon 23 of the mouse DMD gene on the X-chromosome leading to a lack in expression of the protein product, dystrophin. This mouse model is the least severe, most cost-effective and most extensively studied mammalian model of DMD. Mdx mice exhibit no profound behavioural phenotypes, but do exhibit biochemical, histological, mild activity, and muscle electrophysiological changes (e.g., elevated serum creatine kinase, increased muscle regeneration, mild locomotor deficits, reduced specific tetanic force). The mdx mouse is not as severely disabled as the CXMD dog and DMD patient, but it serves as a very appropriate small animal model for testing a range of gene transfer strategies. It is important to note that in our experiments mostly male mdx mice will be used. It has been previously shown (and we verified this experimentally) that while skeletal muscles of female mice present histological defects due to the loss of dystrophin, they show a level of strength comparable to that of wild type mice. Therefore female mice cannot be used to assess functional improvement after muscle treatment and they will be kept primarily as breeders.

DBA2/mdx mouse: these mice are generated by crossing the mdx mouse with the DBA2/J strain that is characterised by extensive fibrosis in muscles. The resulting DBA2/mdx mouse harbours the same stop codon mutation in the exon 23 as the mdx mouse but its muscles also present extensive fibrosis and chronic muscle atrophy, therefore representing a better mouse model of DMD compared with the less fibrotic, pseudo-muscle hypertrophic mdx. This mouse model is particularly helpful when developing strategies to reduce muscle atrophy and fibrosis.

Humanised DMD (hDMD) mouse: this is a transgenic animal made by introducing a yeast artificial chromosome (YAC) sequence containing the full-length human dystrophin gene (hDMD), resulting in expression of a 427 kDa full-length human dystrophin protein. The mouse is generated starting from DBA2 embryonic stem cells but it was successively backcrossed with the C57BL6 for several generations and can now be considered made under this strain. The added levels of human and mouse dystrophin protein are similar to the levels of the murine protein in wildtype mice and therefore these animals breed as wild

type C57BL6 mice, have normal phenotype and show no signs of neuromuscular abnormalities.

hDMD KO mouse: this mouse is generated by inactivating the hDMD locus (by CRISPR/Cas technology in embryonic stem cells) in hDMD mouse and subsequent backcrossing with the mdx mouse model of DMD which does not express murine dystrophin. Therefore this hDMD KO mouse does not express any form of dystrophin. There is no profound phenotype, but these mice do exhibit a number of biochemical, histological, behavioural, and electrophysiological abnormalities, similar to the mild dystrophic phenotype observed in the mdx mouse model, that can be exploited as read-outs for therapies. Importantly, it can be used to develop treatments that are aimed to rescue the functionality of the endogenous human dystrophin expression which represents a major advantage for the translation of such approaches in clinic.

A17 mouse model of Oculopharyngeal muscular dystrophy (OPMD): OPMD is caused by an expanded (CAG) triplet repeat mutation in a protein named Poly(A) protein binding-1A (PABPN1) that is involved in the production of many other proteins in the cells. The A17 mouse is a gain-of-function transgenic model of this disease, generated by genomic inclusion of a short ~1kb long cDNA under control of a muscle specific promoter so that the model expresses the PABPN1 triplet expansion (expPABPN1) in all skeletal muscles. Up to the age of 6 months, these mice exhibit no profound behavioural phenotypes, but do exhibit some muscle atrophy, reduced body weight and biochemical changes. Beyond 6 months of age, the OPMD mouse exhibits progressive muscle atrophy and weakness leading to severe motor dysfunction by 12 months. This is the only animal model available of OPMD that also has a clear phenotype to use in screening for therapies.

New OPMD mouse: While the A17 mouse model is invaluable to screen drugs for OPMD, it cannot be used to test strategies requiring the alteration of the genome splicing. We will generate a mouse where the whole expanded PABPN1 gene (including all exons and introns) is present and expressed in muscle cells. Notably, we expect this mouse to become also useful as further important model for the screening of other more conventional pharmacological and genetic approaches.

Knock-in (KI) mouse model of OPMD: This mouse is generated by breeding B6.Cg-Pabpn1tm1.1Gpvl/J with B6.FVB-Tg(EIIa-cre)C5379Lmgd/J (expressing recombinase CRE). The heterozygous knock-in mouse model contains one alanine-expanded Pabpn1 allele under the control of the native promoter and one wildtype Pabpn1 allele and it is therefore a close genocopy of the human condition. The KI mouse shows less pathological defects in skeletal muscles compared to the myopathy observed in the A17 mouse model probably due to the lower level of expPABPN1 expressed but it is the only murine model of OPMD that is generated starting from the whole expanded PABPN1 gene (i.e. expPABPN1 is present as gene with exons and introns and it is driven by the endogenous promoter). Although the OPMD phenotype in the KI mouse is relatively mild, expPABPN1 expression results in formation of aggregates in the tongue and plantaris muscles develop atrophy which can be used as read-outs of the experiments based on PABPN1 exon skipping.

FSHD Mouse: The pathological sequence by which DUX4 abnormal expression leads to muscle wasting in Facioscapulohumeral dystrophy (FSHD) has been enigmatic. The FLExDUX4 mouse, transgenically generated to carry a full human DUX4 gene, has so far been the most used animal model in research for FSHD as it exhibits remarkable and

important similarity to FSHD-associated phenotypes (i.e. disease-relevant low level DUX4 expression, muscle wasting, muscle weakness, thickening of the outer layer of the skin, alopecia, high frequency hearing deficit), without reproductive issues. When bred to ACTA1-MCM mice (JAX #025750), resulting ACTA1-MCM/FLExDUX4 mice display Cre recombinase-dependent expression of human DUX4 that is induced by tamoxifen. The double transgenic ACTA1-MCM/FLExDUX4 model expresses more DUX4 than the FLExDUX4 model, especially following tamoxifen induction, making this model both conditional and titratable. This allows full control of both the timing of disease onset and the severity, and that facilitates investigations into the mechanisms of FSHD as well as pharmacological therapies for the disease.

Methods includes:

1) The local and systemic (Intravenous or intraperitoneal) administration of agents.
2) Surgery to systemically administer agents by osmotic pump implantation

Why can't you use animals that are less sentient?

Animals used in this project will be at various stages of their development, from newborn to older age, depending on the purpose of each experiment. Mice at this stage of development best represent most aspects of the pathologies we are studying. As the methodologies we are using are mostly genetic approaches designed to demonstrate the efficacy in relevant animal models of the disease, the mouse represents the best option as histopathological analyses, muscle strength and behaviour recapitulate well similar parameters that can be analysed in human. Other simpler models of neuromuscular conditions exist for some of these pathologies (e.g. zebrafish, drosophila, nematodes) but they are not optimal models in terms of preclinical translation. The crucial point is the similarity in physiology and genetic (DNA) content between mouse and human which makes gene therapy and antisense technologies that are effective in mouse also relevant for translation in human.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The measures we will take to minimise harms to the animals include implementation of the 3Rs guidelines, ensuring numbers used are minimised by careful project design and the procedures are carefully refined to ensure severity is as mild as possible. Animals will only be used in our experiments if they are in excellent health. They will be housed in conditions of very high supervision and welfare. They will be supervised by skilled and caring technicians on a daily basis and supervised regularly by an independent veterinary surgeon. The animals will be housed communally and in enriched environments to maximise social, welfare, rehabilitation considerations. All animals will be humanely culled using schedule 1 procedures.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The ARRIVE guidelines will be followed to design the experimental plan for our experiments. Following ARRIVE guidelines is now required by most of the funders when submitting grant applications and when performing experiments. They are particularly important as they help to design the best experimental procedures for these types of

studies. For example when working with ARRIVE guidelines:

It is important to explain the study design in great detail, so that all essential information on the procedures done in animals are described including the use and the rationale of adding specific positive and negative control groups.

Information on animals like species, strain, sex, weight, and age must be disclosed and the rationale of these specific choices need to be explained.

The calculation behind the number of animals/group needs to be properly justified using specific software for the power calculation (e.g. GPower, PASS and similar programs) or by consulting statisticians every time it is needed.

Before starting the procedures, specific inclusion/exclusion criteria are set based on previous experience within similar experimental conditions to maximize the welfare of animals and make reliable and statistically solid the dataset generated.

It is crucial to avoid any bias during the analyses, which is obtained by both randomizing the animals before starting the experiments (e.g. by dividing mice from a litter between multiple groups and conditions) and by working in blind so that the operator doing the analysis cannot associate the animal to specific treatments/conditions.

Outcome measures are similarly defined before starting the experiments. The most suitable read-out must be chosen and each of them has to be measurable. This allows to use statistical tools to analyse data so that results provide reliable results and can truly inform on the success of the experiment.

Finally, the data generated by these experiments must be reported in manuscripts or through other forms so that they become publicly available and can inform other experiments and studies.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We check the NC3Rs website on a regular basis and maintain constant communication with the staff working in our animal facility, the Named Veterinary Surgeon (NVS) and other scientists using animals for research. This ensures we are kept up to date with the latest news so that we can implement any necessary change timely and effectively.

76. Arginine methylation and tumourigenesis

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

cancer therapy, cancer development, PRMT, cancer stem cells, DNA repair

Animal types	Life stages
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult, Aged animal

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Protein arginine methyltransferases (PRMTs) are a family of proteins called enzymes that regulate the function of cells by adding a chemical group onto the target protein. Recently, it has been shown that PRMT expression and activity is high in breast and lung cancer, and a children's cancer called Ewing Sarcoma. Studies using cells in the lab have provided strong evidence that drug targeting PRMTs in these cancer types could be an important new therapeutic approach. Because of this there are now drugs to PRMTs in clinical development. However, we still do not fully understand how PRMTs promote cancer or drug resistance, particularly in context to the whole organism. Understanding this is essential as it will enable us to understand how PRMT inhibitors can be successful used in the clinic to treat cancer patients.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This project focusing on the role of PRMTs in breast and lung cancer, and in Ewing Sarcoma:

Breast cancer: Every day, 31 people die of breast cancer in the UK resulting in 11,400 deaths per year. Breast cancer is therefore the 4th most common cause of cancer death in the UK, accounting for 7% of all cancer deaths. Breast cancer is a highly complex disease with many subtypes that require different drug treatments. We therefore need to understand how breast cancer develops and avoids treatments to find new therapies.

Lung cancer is the third most common cancer in the UK with around 48,500 people are diagnosed in the UK each year. Only 15% of patients will survive their cancer for 5 years or more. This highlights the needs to find new ways in which to treat patients.

Ewing sarcoma is a rare cancer of bone and soft tissues affecting children and young adults. Standard of care involves chemotherapies, radiotherapy and surgery however 30% of patients will not be cured. Sadly, those whose disease has spread or comes back after treatment have a very poor outcome. As treatments are given to children/young adults, co-morbidities (e.g. sterility) and other cancer later in life are common.

For breast cancer, lung cancer and Ewing sarcoma patients, the drugs that are administered target cancer cells that are dividing rapidly. However, this approach has many unwanted side effects as normal dividing cells can also be killed. Furthermore, cancer cells often become resistant to these drugs meaning that tumour might grows back. The discovery that some genes when mutated or expressed at abnormal levels can drive specific cancers has led to a more targeted approach that means fewer unwanted side effects and a reduced chance that the cancer comes back. This is called rational drug design. This strategy has only been made possible by our increased understanding of the mechanisms underlying cancer and our ability to test new drugs in a whole animal model. Scientists have made important new discoveries using this approach, however as cancer is incredibly complex there is still a continuing need to identify and test novel therapeutic targets ensuring that as many patients as possible lead happy and healthy lives.

What outputs do you think you will see at the end of this project?

The principal outputs from this project will be new information that will be disseminated to the pharmaceutical and academic community via high impact publication and presentations at conferences. We expect to show that genetic and/or pharmacological disruption of PRMTs reduces the growth of breast, lung, and Ewing sarcoma cancer which will greatly support the development of clinical trials using these inhibitors in human patients. We will also show that combining PRMT drugs with other drugs is a way in which to use lower concentrations but still see a good effect on stopping cancer growth. In patients, this should mean that fewer side-effects are observed.

We will understand which genes that are known to be abnormal in cancer can work with PRMTs to drive cancer growth providing a mechanistic understanding into how PRMTs facilitate cancer formation. This will enable us to identify patient groups that will respond well to PRMT-directed therapies. We will also demonstrate the importance of PRMTs in keeping breast cancer stem cells alive. This is important because breast cancer stem cells are a special type of cancer cell. Whilst it only makes up a small proportion of the total

tumour cells, it is known to promotes drug resistance and the spread of disease to other organs. Drug targeting this special cancer cell type is thus of upmost clinical importance.

We will also examine in the whole animal the importance of specific proteins whose activity is modified by PRMTs. Again, this will provide a mechanistic understanding into how PRMTs facilitate cancer formation but also identify biomarkers that might indicate which patients will respond well to PRMT therapies.

Taken together, our outputs could be rapidly expedited into clinical trials. For example, in Ewing Sarcoma, we already have links with pharmaceuticals that have PRMT inhibitor in phase I clinical trials for adult cancer. This, coupled with our collaborations with clinicians that run paediatric clinical trials, means our results could rapidly lead to phase I clinical trials of PRMT inhibitors in this treatment of this childhood cancer.

Finally, we want to develop an alternative approach to the classical way in which to genetically model breast cancer by using CRISPR/Cas9 technology, often referred to as "molecular scissors". Currently, genetic mouse models are generated through very complex breeding schedules that require genetic changes in the germline of the animals. In contrast, we want to use viruses that contain these new molecular scissors and introduce them directly into the mouse mammary gland. This simplified model will provide us with a method in which to induce several different genetic events using one biological tool thereby reducing animal numbers. We hope that by validating this model in the context of PRMT biology, this simplified and reductive approach will be taken up by others in academia and industry thereby further accelerating new research findings.

Who or what will benefit from these outputs, and how?

This project will greatly advance the biological understanding and impact of PRMTs on disease processes and is most likely to be of interest to pre-clinical scientist interested in tumour biology. A second benefit will be the validation that modelling breast cancer in mice can be achieved with a much less complex approach, significantly facilitating the ability for researchers in the breast cancer community to investigate their gene of interest in a way that involves the use of fewer animals. The third benefit is the pharmaceutical sector that are developing small molecule compounds targeting PRMTs. These three outputs are expected to be achieved during the lifetime of this project. The fourth long-term benefit of this project is to cancer patients. This most likely will be realised towards the end of this project where we can demonstrate that genetic and chemical modulation of PRMT activity restricts tumour growth, and that combination therapy with other agents is effective in reducing tumour growth. Our mechanistic understanding could lead to ways in which to stratify which patients will respond to treatments, and therefore, in the long-term could lead to a change in clinical practice.

How will you look to maximise the outputs of this work?

We are already collaborating with several prominent academic and leading industrial collaborators that will help facilitate the dissemination of our findings. We will present our findings in a timely manner at conferences and symposiums. If we are successful in modelling breast cancer using the "molecular scissors" technology, we will request funds to hold a workshop to demonstrate this technology to others that are interested.

I am also actively involved in disseminating our research to supporters of cancer charities

through presenting our research and hosting lab tours. Whilst talking about animal experimentation can be a contentious subject, engaging with the public provides an excellent opportunity to discuss the 3R principle and scientific studies involving animals in the UK to broaden perspective.

Whilst it is still challenging to publish unsuccessful approaches, it is important that this information is disseminated to prevent serendipitous repetition and so we will endeavour to include negative results in our publications.

Species and numbers of animals expected to be used

- Mice: 4200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We use mice to enable a genetic approach to understand how cancer develops and thus identify novel targets for therapeutic intervention. We also use mice to determine if drugs that target a protein or cellular pathway that we have identified using culture cancer cell lines are effective in treating tumours in the whole organism.

Mice are the species of choice because there are a large number of widely available genetically modified strains that means that the function of most genes of interest can be studied. Furthermore, they can grow human tumours as xenografts enabling drug testing and in vitro genetic manipulation before implantation. This enables us to mechanistically interrogate tumour growth and identify new way in which to drug target this.

We are predominantly using mice strains that are conditionally altered for our genes of interest using Cre technology. This means that genetic events will only occur in a specific tissue minimise the effect of genetic alteration on the whole animal. Whilst some Cre lines are predominantly active in adult tissue, some are switched on during embryogenesis, and this can lead to embryonic lethality if a gene of interest is critical for this process. To overcome this, we are using a method to induce genetic recombination in the adult mouse through the delivery of Cre-expressing viruses to a specific organ.

A second reason we have chosen to use mice is that most cancer models we will be using have been extensively characterised previously and develop tumours with similar aetiology and molecular profile as that observed in human disease. Tumour growth rate is predictable, thus fewer mice are required to be used for each experiment. Some of our studies will involve the generation of novel genetically modified animals. In all cases, we will closely monitor animals and ensure that their welfare is always considered.

Several of our approaches require the growth of human cancer cell lines or patient-derived tumours (PDX), and this must be conducted in immune compromised mice to prevent human cell rejection. We choose to use adult mice of a specific age range (5-7 weeks of age) to minimise variability.

In some cases, we need to isolate cells from mice that have undergone genetic manipulation. This may involve using embryonic or post-natal cells in which the genetic alteration can be induced post-mortem in the cells directly. This provides us with a source of genetically altered cells in which we can conduct in vitro experiments, including mechanistic studies.

Typically, what will be done to an animal used in your project?

Most animals on this licence will develop tumours. Some animals may be born with genetic alterations that pre-disposes them to develop cancer whilst others will need the genetic event inducing by delivering a compound to the mouse. Other animals will undergo implantation of human or mouse cancer cells under the skin on the back of the animal leading to tumour development at this anatomical location. Some of the cancer cells we inject will require additional hormone to be present to enable tumour growth, hence these animals will undergo surgical implantation of a hormone pellet under the skin (under general anaesthesia). This means that we do not need to subject the animal to repeated injections. In some cases, tumour growth can be monitored though non-invasive bioluminescence imaging involving injections of luciferin and general anaesthesia. Most animals will have tumour growth rates measure by non-invasive calliper techniques.

Other animals will need to develop tumours within the mammary gland so that tumours are growing in an environment that best mimics human disease. In some cases, we will inject cells or pieces of tumours into or close to the mouse mammary gland. In other cases, we will use the MIND (**M**ouse **IN**tra**D**uctal) model. Here, breast cancer cell lines are injected into the mammary ducts leading to the development of disease that more closely resembles that found in patients. Alternatively, we will genetically engineering mice that leads to the expression or deletion of specific genes that predisposes a mammary gland cell to cancer development. This will be achieved through either through conventional germline breeding strategies or by using CRISPR/Cas9 "molecular scissors". This latter model involves the delivery of viral particles to the ducts of the mammary gland.
Around half of the animals growing tumours will be administered with drugs to determine the effects of small molecular inhibitors that target PRMTs, either alone or in combination with DNA damaging chemotherapies. Here, the route of drug administration depends on the specific agent, but could include intraperitoneal, intravenous or subcutaneous injection, or by oral gavage. To show that the DNA damaging drugs can inhibit their target protein in a mouse, mice might undergo whole body irradiation to induce a DNA damage response and humanely killed within 24 hours. To check that chemotherapies are not adversely affecting mice, we will take tail vein blood samples to test for anaemia and leukopenia. In some cases, when we see a good tumour response after a course of treatment, some animals will be taken off drugs and allowed to age to examine long term effects of drug withdrawal. Here, the critical question is whether tumours grow back after time.

We are also wanting to know the effect of inhibition or genetic manipulation of PRMTs on normal mammary stem cell function. To do this, some female mice that have undergone gene deletion by conventional germline genetics, or via introducing substances to the ducts of the mammary gland, or via transgenic overexpression through genetic means, will be inseminated through natural timed breeding. Mice will be injected with BrdU and humanely killed before birth of the litter. We are then able to measure pregnancy-induced proliferation post-mortem as this is a marker of mammary gland stem cell function.

What are the expected impacts and/or adverse effects for the animals during your project?

Many of the mice we are using will be genetically pre-disposed to generate tumours or will be engrafted with human/mouse cells that will form tumours as a xenograft. Hence, the main adverse effects will be related to this. Animals will be humanely killed if they demonstrate weight loss (maximum of 15%), or a BC score of <2, or reduced activity, starey coat and/or hunched posture for 4 hours or more, or if cumulative tumour volume reaches 12mm in diameter, or 1.25cm2. In the case of mammary tumours developing within the mammary gland, there is a small chance that tumours may ulcerate. In the event of this, the animal will be humanely killed.

It is possible that delivery of chemotherapeutic drugs will cause adverse effects. As the drugs we will use as well reported in the literature, we will be able to pay close attention to the development of any signs of distress. This might include anaemia, leukopenia and gastro-intestinal disturbances. Anaemia and leukopenia can be monitored by blood analysis. Repeated treatments with chemotherapeutics may cause a chronic decrease in body weight between 5-15% over time as well persistent diarrhoea, lethargy, and abnormal breathing.
In all procedures, mice will be closely monitored and welfare recorded throughout using a welfare sheet.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Sub-threshold: 30%

Mild: 10%

Moderate: 60%

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Very few, if any, cell culture-based models that we use in the lab can recapitulate the complex interplay between cancer cells, normal cells (stroma, immune cells, blood vessels), or the metabolic conditions that occur in a tumour within a whole organism. Consequently, studies using standard in vitro cancer cell lines alone are less able to predict outcomes in the whole organism. Importantly, genetic analysis of gene function

during the cancer process has been essential for the development of novel therapies for this disease, and generally involves the genetic modification of mice that demonstrate in a whole organism its significance for cancer growth or drug resistance. Drug testing of preclinical/clinical compounds, either as a single agent or in combination with other agents, in tumour-baring animals is required before initiation of human clinical trials.

In this project, we will use multiple mouse models to fully address our scientific questions. This means we can fully consider their relevance to human disease (i.e. the use of patient-derived tumour material) and the importance of the immune system in in tumour development and drug treatments (i.e. the use of genetic mouse models and the use of mouse cancer cells lines that can implanted back into a mouse). Importantly, as we can genetically modified cancer cell lines in the lab, we can use this tool to look specifically at the effect of one mutation of a gene of interest on cancer growth in the mouse in a controlled way. Together, these multiple models will make our work more impactful for the patient.

Which non-animal alternatives did you consider for use in this project?

In vitro data obtained from cell culture approaches will always guide in vivo studies, and we always conduct cell culture-based experiments to justify the need to use animals. These systems include culturing cells in monolayer as a homogeneous population on plastic, and more sophisticated 3D cell cultures including acinar growth in Matrigel (as acinar) or mouse/human derived tissue as organoids. Additional alternative systems we will use if available include in silico analysis of tumour pathways, either in response to drug treatment or after removal or a protein component.

Why were they not suitable?

There are significant limitations of using cells grown in isolation as a monolayer on a piece of plastic as this is not an accurate representation of what occurs within a patient. Indeed, the reason why many new drugs fail between cell culture and in vivo studies is in the inability to full recapitulate the in vivo environment (non-tumour cell component, blood supply, metabolic conditions). Technologies are being developed to address this gap, including the development of 3D cultures (acinar cell line cultures and organoid mouse/human derived tissue) that can recapitulate some metabolic conditions (i.e. hypoxia at the tumour core) and rudimental tumour 3D structure. However, the further development of these model systems to accurately phenocopy the interplay between the numerous cell types that constitute a tumour and its microenvironment is still lacking. Likewise, the ability to genetically manipulate patient-derived organoid cultures is still technically challenging meaning that the experiments that can be performed are restrictive. In silico prediction of the effects of genetic manipulation/inhibition of PRMTs, or the consequence of expressing substrates that cannot be targeted by PRMTs is not possible because we cannot predict the unknown.

Modelling cancer in mice is thus still required to fully understand disease progression and identify novel therapeutic avenues.

Reduction

Explain how the numbers of animals for this project were determined. Describe

steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Animal numbers will be based on previous in-house data where it exists, otherwise we will use published data, that from collaborators, or undertake pilot studies to obtain the required baseline data to perform a calculation to provide the animal numbers.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All of our experiments have been designed with the aim to reduce animal numbers but without compromising the scientific objective. For example, if suitable, we have used the NC3Rs EDA in conjunction with a local statistician to assist with experimental design to reduce animal numbers, and couple this with good experimental design to reduce subjective bias. We also have used the appropriate statistical analysis to ensure as few animals as possible are used without compromising the scientific objectives. When possible, experiments have been designed to be factorial therefore maximising the information obtained from an animal. For example, we often use live imaging (e.g. IVIS) and calliper measurements to track tumour development longitudinally meaning that less animals are needed overall as there is no need to humanely killed at each time point. Moreover, variation is reduced because data is produced as multiple measurements on the same animal over a period of time. In such cases, ANOVA will be utilised for statistical analysis.

When using syngeneic models, we try use the same mouse strain in which the cell lines was derived from (e.g. 4T-1 and Balb/c mice) to maximise the rate of engraftment.

The breeding strategies are designed to obtain the required genetically altered animals with the minimum amount of wastage, and where possible littermates will be used as controls. This not only reduces wastage directly, but also ensures a more accurate comparison to the experimental animal which means less animals are required.

To calculate frequencies of stem cells in cancer cell populations, the L-Calc program is used. Five dilutions into five animals are required for robust data with smaller 95% confidence intervals.

All strains not immediately required for scientific study will be cryopreserved.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We always strive to generate the most effective breeding strategies to ensure that we obtain mice of the desired genotype with minimal animal wastage. If we are unable to estimate an effect size from our in vitro data, the literature, or our collaborators, we will conduct small pilot experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

For most cases, we have chosen genetic mouse models that will develop spontaneous tumours that have been extensively characterised, hence, we will be aware of potential adverse effects and monitor for them appropriately. The phenotype of any novel genetically modified strain will be closely monitored using a welfare sheet to identify potential adverse effects at the earliest possible stage. When using tumour models that spontaneously develop breast cancer, we avoid using females that are genetically predisposed for tumour development for breeding as pregnancy is known to decrease tumour latency. In this case, the male in the breeding pair carries the cancer-causing gene.
When we are conducting transplantation experiments with a cancer cell line for the first time, we will conduct a small pilot experiment (n=3 mice) to monitor growth rates and potential adverse effects such as tumour ulceration. This information will be used to inform on timing and welfare intervention. Where possible, monitoring of engrafted tumours will occur through non-invasive techniques (bioluminescence or calliper measurements) that will enable us to track tumour progression in living animals throughout the experiment. In some cases, we may need to conduct a pilot experiment (n=3 mice) of a new drug, or a new batch, in non-tumour bearing animals to ensure target engagement and functionality before commencing with a procedure that requires a larger cohort of animals that are developing tumours.

The MIND (**M**ouse **IN**tra**D**uctal) model offers a refinement in the study of ER+ breast cancer as ER+ breast cancer cell lines will grow in systemic levels of estrogen therefore alleviates the need of surgical implantation of hormone pellet.

Why can’t you use animals that are less sentient?

We have chosen to use mice over other less sentient species such as Danio Rerio (zebra fish) and drosophila melanogaster (the fruitfly) as mice and humans share 97.5% of their coding DNA sequences. In comparison, the drosophila genome is only 60% homologous to that of humans and only 75% of the genes responsible for human diseases have homologs in flies. Mice are also a more appropriate for studying complex biological systems found in humans as they possess immune, endocrine and nervous system. Terminal anaesthetised animals are not appropriate for the study of cancer development due to length of time required for tumours to grow, or the time duration required (weeks) to see an effect of a drug.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

When conducting a surgical procedure, we ensure that analgesia is administered pre-surgery with use of post-surgery support and monitoring (i.e, keeping animals warm and under close observation). Sometimes wounds will be closed by both suturing and glue to minimise wound re-opening.

Once mice start to develop tumours, they are monitored at least twice a week, and more so if tumour growth develops rapidly. For animals in which tumours develop within the mammary gland, hard housing is replaced by cardboard housing and additional bedding to prevent ulceration.

We are highly trained in intraductal injections and members of my team undergo extensive training on cadavers before being allowed to perform a procedure on live mice. We have found removal of fur in this area via cream was more comfortable for the animal post-recovery than using the clippers. We also generally prefer to inject the 4th gland as it is furthest from the armpit so less likely to impact on mobility/comfort of the animal.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Guidelines for the welfare and use of animals in cancer research. Workman P, Aboagye EO, Balkwill F, Balmain A, Bruder G, Chaplin DJ, Double JA, Everitt J, Farningham DA, Glennie MJ, Kelland LR, Robinson V, Stratford IJ, Tozer GM, Watson S, Wedge SR, Eccles SA; Committee of the National Cancer Research Institute. Br J Cancer. 2010 May 25;102(11):1555-77. doi: 10.1038/sj.bjc.6605642.

RSPCA and LASA, 2015, Guiding Principles on Good Practice for Animal Welfare and Ethical Review Bodies. A report by the RSPCA Research Animals Department and LASA Education, Training and Ethics Section. (M. Jennings ed.)

An applied approach to assessment of severity. In: Humane End points in Animal Experiments for Biomedical Research (Hendriksen CFM, Morton DB, eds). Jones HRP, Oates J, Trussel I BA (1999). London: Royal Society of Medicine Press, pp 40±7.

LASA dosing guidelines:
https://www.lasa.co.uk/PDF/LASA-NC3RsDoseLevelSelection.pdf

Jones HRP, Oates J, Trussel I BA (1999) An applied approach to assessment of severity. In: Humane End points in Animal Experiments for Biomedical Research (Hendriksen CFM, Morton DB, eds). London: Royal Society of Medicine Press, pp 40±7. http://www.lal.org.uk/uploads/editor/HEP_JONES.pdf

We will publish in journals that support the ARRIVE guidelines and conduct our experiments with advice from the PREPARE publication (PREPARE: guidelines for planning animal research and testing. Smith AJ, Clutton RE, Lilley E, Hansen KEA, Brattelid T. Lab Anim. 2018 Apr;52(2):135-141. doi: 10.1177/0023677217724823. Epub 2017 Aug 3.PMID: 28771074).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

My lab will comply with the ARRIVE guidelines (Animal Research: Reporting In Vivo

Experiments; www.nc3rs.org.uk/arrive), a NC3Rs-developed checklist of the essential information that should be included in publications reporting animal research. ARRIVE has now been endorsed by more than 400 journals including the Nature group, PLoS, and Cell, as well as funders, universities, and learned societies.

I have subscribed to the NC3Rs newsletter, so are made aware of any notification at the earliest possibility. Any new advancements will be made clear to members of my team through our weekly lab meetings.

77. Improving outcomes following nerve injury and repair

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Nerve injury, Nerve guidance conduits, Neuropathic pain, Nerve regeneration

Animal types	Life stages
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult
Rats	Adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overarching aim of this programme of work is to determine an optimal therapeutic/surgical intervention to enable improved peripheral nerve regeneration and reduce neuropathic pain following injury.

The three key elements of this are:

1. Improve functional regeneration through the use of therapeutic agents that act to modulate the cellular response at the site of nerve injury.
2. The creation of nerve guides with an active influence on the guidance and regeneration of injured nerves.
3. To further our understanding of the mechanisms underlying neuropathic pain and identify interventions that may reduce this pain.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Background: Approximately 300,000 peripheral nerve injuries arise each year in Europe; predominantly via work, domestic and traffic accidents. Direct microsurgical repair is the mainstay of treatment, with end-to-end suturing of the two nerve ends the current optimal method, however significant movement and sensory deficits often remain following treatment. This method of microsurgical repair is also not appropriate for injuries where the injury has produced a gap in the injured nerve.

Longer injury gaps are usually bridged with a nerve graft taken from a different nerve in the same patient, this may provide some functional recovery, but recuperation of muscle movement is poor. There is also a major disadvantage principally related to the need to retrieve donor material from the patient, leading to further loss of function and sometimes pain at the donor site. Furthermore, insufficient graft material is available for major reconstruction and therefore the future direction of nerve repair is the creation of bioengineered nerve grafts. However, modern day nerve guides are not designed to actively stimulate regeneration processes necessary for optimal nerve recovery and consequently rarely support regeneration over distances greater than a few millimetres.

In addition to loss of motor and sensory function a large proportion of patients (~50%) have persistent pain for which there is no reliable treatment [1]. This is associated with sleep deprivation, depression and adverse psychological problems. Significant prolonged disability, plus socio-economic dependency on family and social services is inevitable. Of patients with injuries in the forearm less than two-thirds return to employment.

The poor outcomes reflect microsurgical failure to address nerve regeneration at either a biomaterials device or cellular level. Our project will address these gaps as indicated by the aims specified above.

This project aims to improve the outcomes following nerve injury, both in terms of nerve function and neuropathic pain. It is of considerable clinical relevance and in relation to clinical work at our centre, there is a translational route to patient treatment via our provision of a national centre for nerve repair. Our particular clinical expertise relates to injuries to the nerve that supplies the face and mouth. These injuries have a profound effect on a range of normal everyday activities and have a considerable impact on quality of life. They may occur during wisdom tooth removal, the placement of dental implants or corrective jaw surgery, or as a result of facial fractures. Following nerve injury, a proportion of patients are left with complete numbness or altered sensation of the affected region; this may be accompanied by severe nerve injury-induced pain (neuropathic pain), which is often described by patients as stabbing or burning in nature. Regions most commonly affected are the lips and tongue. Numbness and pain in the affected areas can affect the ability to speak and eat, leading to anxiety, depression and isolation. Prior to our previous and ongoing research and the establishment of our centre little had been offered to these patients by standard clinical practice, either in terms of a clear prognosis or possible surgical intervention to improve the level of recovery.

Research at our centre over many years has answered a series of fundamental questions regarding the efficacy of repair using various repair procedures. The research has enabled identification of the most successful repair techniques, and the cases likely to benefit from surgery and those that will not. This has reduced surgery in cases where it is not likely to be beneficial. It has also ensured that where surgery is indicated it is carried out by surgeons specifically trained in nerve repair, using evidence- based approaches that give the best chance of success. The work has also enabled provision of information to patients on likely outcomes and potential risks and benefits.

The repair service feeds back into our research on neuropathic pain. The repair procedure includes the removal of the ‘neuroma’, a mass of tangled sprouting axons and scar tissue that develops at the site of nerve injury. These neuromas are used in our neuropathic pain research and have helped in the understanding of mechanisms underlying neuropathic pain.

Our bench to bedside approach is an excellent example of the translation of in vivo research into clinical practice.

What outputs do you think you will see at the end of this project?

Outputs from this project will include publications in peer reviewed journals, development of new methodologies for advancing nerve repair, knowledge related to the development of chronic pain - particularly that occurring as a consequence of nerve injury (known as neuropathic pain), identification of novel potential therapeutic targets with potential for development of novel drugs.

Who or what will benefit from these outputs, and how?

The programme has considerable potential to improve treatment for patients with nerve injury and neuropathic pain. Improvements may include improving nerve repair for cases where there is a large gap in the injured nerve (through the use of bioengineered nerve guidance conduits), and improvements in the treatment of neuropathic and other forms of chronic pain, via the identification of novel therapeutic targets potentially facilitating drug discovery. Our current work is supported by funding from UKRI and the pharmaceutical industry, and this will expedite drug development arising from this project.

Drug development is a lengthy process, so development of novel therapies is not likely in the course of the project. There is potential that improvements in nerve repair procedures may reach clinical trial within the time frame of this project.

Potential benefits to patients include novel interventions to tackle debilitating peripheral nerve injures and neuropathic pain, via reduction of motor and sensory disturbance, reduction of neuropathic, and associated disability. Leading to improved quality of life and alleviating the societal and economic impact of these injuries.

How will you look to maximise the outputs of this work?

Our findings will be disseminated at academic and clinical conferences and published in peer reviewed journals.

Our collaborations with the pharmaceutical industry will facilitate drug development arising

from this project.

Our centre provides a national referral service for nerve repair, and the presence of experienced clinical staff within our research group who treat patients referred for nerve repair, put us in an excellent position to translate our research to the clinic.

Species and numbers of animals expected to be used

- Mice: 1000
- Rats: 200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The physiological response following a peripheral nerve injury involves a complex series of events, with many interactions that are as yet not fully characterised, including events occurring in the central nervous system (the brain and spinal cord). As our goal is to improve the overall outcome of peripheral nerve injuries - through the application of therapeutic agents or nerve guidance conduit - we require a live animal model to assess the full range of effects and efficacy within the nervous system, which cannot be predicted using isolated tissues in vitro.

These studies will be carried out in adult rats and mice. We have no reason to expect any significant species differences in nerve regeneration between rodents and man, but there are differences in regenerative abilities in lower order species such as reptiles. We therefore believe that rodents are the appropriate choice for these studies, which is consistent with the majority of previous investigations.

The majority of the work intended to be performed under this licence can be achieved using mice; however, due to size constraints mice are unsuitable for studies investigating the nerves supplying the mouth and face, or those investigating extended nerve defect lengths. Therefore, we believe that in experiments investigating nerves supplying the mouth and face, or peripheral nerve regeneration over relatively long distances, the choice of the rat is justified.

We will use thy-1-YFP-H mice, these mice are genetically altered and a subset of their neurons are fluorescently labelled. We have used this strain in our nerve injury and repair studies for several years. The mutation is non-harmful, and the fluorescent axons present in their nerves provide considerable benefit over wild-type mice when visualising and quantifying nerve degeneration and regeneration.

Typically, what will be done to an animal used in your project?

Some animals will undergo nerve injury with or without or nerve repair procedures (under general anaesthetic). During these procedures an incision will be made in the skin and the relevant nerve will be exposed and injured. If the nerve is repaired, this will be either by

direct end-to-end suturing, or with a nerve graft or with a nerve guidance conduit secured in place using fibrin glue or sutures. In some cases animals (with or without repair) will also be treated with a range of agents that may improve nerve regeneration and/or influence the development of neuropathic pain. The agents to be used will look to reduce inflammation/scarring or enhance levels of factors beneficial to regenerating nerves at the site of injury. Animals may be allowed to recover for a period of up to 6 months, but in most cases it will be no more than 6 weeks. Animals providing nerve grafts only will be non-recovery.

Some animals will be used to investigate the effects of nerve injury on tissue healing. In these animals wounding of the ear pinna (via a small ear punch - as is commonly used for identification purposes) will be carried out in animals with a nerve injury that affects the nerves supplying the ear pinna. Control mice will have only the ear punch carried out. Animals will be allowed to recover for a period of up to 6 weeks post-wounding.The outcomes will be used to inform future studies that will look to develop potential therapeutics for treating chronic wounds, including those that are associated with conditions where the peripheral nerves have been compromised by nerve injuries and disease (such as diabetes).

The final procedures will be undertaken under non-recovery anaesthesia where the animals will only be aware of the anaesthetic being administered and may briefly experience distress and no pain. These procedures may include electrophysiological recording and/or harvesting of tissue.

Some animals will be used for breeding to provide genetically altered (GA) mice with a subset of fluorescent nerve axons. We have worked with this strain of mice for many years. The mutation is non- harmful, and the fluorescent axons present in their nerves provide considerable benefit when visualising and quantifying nerve degeneration and regeneration. Animals will be used to produce and provide GA mice via conventional breeding methods. In some cases tissue samples may be taken to determine genetic status, this is achieved by standard ear notching procedures. Offspring may be retained for breeding or utilised for other procedures.

What are the expected impacts and/or adverse effects for the animals during your project?

The animals who undergo nerve injury will experience some immediate and transient post-operative pain, and longer term some loss of function. The loss of function will depend on the nerve that is injured. The deficits will be in sensation and movement in the hind limb, or sensation in the mouth and face. These effects will be present until the peripheral nerves have regenerated, however this is a very slow process so in most cases where recovery is 6 weeks or less, the deficits will be present throughout the recovery period.

Rarely sutures may be lost during recovery. If this leads to wound opening wounds will be closed with tissue glue. This procedure can be carried out while the animal is conscious. If re-suturing is required, animals will be briefly anaesthetised for this procedure.

Animal undergoing nerve surgery will experience some post-operative discomfort and will be given analgesics.

Animals used for breeding will experience minor transient discomfort following ear notch

(punch) procedures, and do not require analgesics.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Animals providing nerve grafts: non-recovery.

Animals undergoing nerve injury with or without repair: moderate

Animals used for breeding: 90% sub-threshold, 10% mild

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The physiological response following a peripheral nerve injury involves a complex series of events occurring in both the peripheral nerve and the central nervous system (brain and spinal cord), with many interactions that are as yet not fully characterised. As our goal is to improve the overall outcome of peripheral nerve injuries through the application of therapeutic agents or conduit guidance - we require a live animal model to assess the full range of effects and efficacy within the nervous system, which cannot be predicted using isolated tissues in vitro

Which non-animal alternatives did you consider for use in this project?

Consideration has been made regarding the possibility of using in vitro tissue explants for this work; however, as mentioned above, in vitro experiments cannot yet replicate the full range of effects and interactions that take place within an in vivo environment following nerve injury. We routinely test the suitability of materials used to make our nerve guidance conduits using in-vitro cultures of a range of cell types. This allows us to assess any potential toxic effects of the materials and their potential for supporting nerve regeneration. As described in the background section above, the clinical service for nerve repair (that has been developed as a consequence of our previous in-vivo work), provides us with access to unique human tissues with linked clinical pain data. This tissue is used in our neuropathic pain research and gives us considerable insight into neuropathic pain mechanisms in man. These tissues have been used in a number of studies and have identified a series of proteins and microRNAs whose expression is linked with presence and severity of neuropathic pain. It is hoped that this research will identify novel targets for neuropathic pain, and this area of research is currently being taken forward through an industry/research council impact acceleration award and an industry/research council PhD

studentship award.

Why were they not suitable?

The initial in-vitro work described above can provide valuable data regarding the suitability of novel materials and provide some indication of whether these materials have any toxic effects and are capable of supporting nerve growth (needed for nerve regeneration) in the very short term. However, they do not allow assessment of how the final conduit will function to support regeneration over the time required for nerve regeneration in a living animal. In the in-vivo situation, success of nerve regeneration and the likelihood of any repair process inadvertently generating neuropathic pain is influenced by many factors and complex interactions between numerous cell types within the peripheral and central nervous system. Thus, it is not possible to assess nerve regeneration and development of neuropathic pain in an in-vitro system.

In relation to furthering our understanding of the mechanisms underlying neuropathic pain and identifying interventions that may reduce this pain; as described above we can identify molecules linked with the development and severity of pain using human neuromas removed at the time of nerve repair. However we cannot assess the efficacy of interventions to reduce or prevent the development of neuropathic pain using these tissues.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Cytocompatibility of all materials used in nerve conduit construction will be assessed in vitro using cultured neurones and glial cells prior to any use in vivo.

Animal numbers have been calculated based on our previous data from similar studies.

We estimate that protocol 1 will use up to ~720 mice; it is likely that due to the combination of conduit designs and different doses of regeneration enhancing agents to be assessed, along with positive controls (eg graft repairs) and other controls (eg simple hollow conduits, exposure of nerves with no injury), and assessment at different time points, we estimate a maximum of ~85 groups of 8 mice will be required overall, plus additional mice (~40) for pilot work.

Groups of 8 should enable differences of approximately 4% in axon disruption, 16% in unique axon regeneration, and differences in compound axon potential ratio of 0.21 to be detected using our established analysis method for assessing regeneration. Whenever possible we will seek advice from an experienced statistician with regards to using factorial experimental designs in order to attempt reduce the group sizes without lessening the statistical power of the experiments. **Total for protocol 1 = 720 mice**

Protocol 2 will use up to 160 rats; 80 for spinal nerve experiments [10 groups of 8] and 80 for trigeminal nerve experiments [10 groups of 8]. **Total for protocol 2 = 160 rats**

Protocol 3 will use up to 130 mice and 40 rats. The mice may be surplus wild-type or YFP+ mice from the thy 1 YFP-H breeding colony, or suitable alternatives, and will be used to provide graft tissue, and cells for in-vitro work. Surplus tissue may be offered for use under recognised research programmes. **Total for protocol 3 = 130 mice and 40 rats**

Protocol 4 is exclusively for breeding and maintenance of our thy-1-YFP-H mouse colony, the estimates of numbers for this protocol is based upon observations of the frequency and sizes of recent litters, ~6 mice per litter, from 5 breeding pairs and 6 litters per year per pair, will produce ~**900 mice in total**. A minimum of 5 breeding pairs will be used per year, which equates to **~50 mice for breeding** over the period of the licence, the remaining mice will be allocated for use under this licence, or made available to other project licences authorised to use them, or culled for tissue for use under recognised research programmes

The current breeding arrangements should provide sufficient YFP+ mice at regular intervals to enable the desired programme of work to be achieved; however, if an individual study requires a greater number of age matched mice in a relatively short time period than can't be provided by this arrangement, the number of breeding pairs may be increased.

Before each experiment is conducted, a detailed protocol will be written covering: (i) a statement of the experimental objectives; (ii) a description of the experiment, covering such matters as the experimental treatments, the size of the experiment, and the experimental material; and (iii) an outline of the method of analysis of the results.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will use the principles of the NC3R's Experimental Design Assistant in our experimental design. Before each experiment is conducted, a detailed protocol will be written covering: (i) a statement of the experimental objectives; (ii) a description of the experiment, covering such matters as the experimental treatments, the size of the experiment, and the experimental material; and (iii) an outline of the method of analysis of the results. Factorial designs are preferred, and power analysis is used where appropriate.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

As described above initial assessment of nerve guidance conduits, eg to assess toxicity of materials and their ability to support nerve outgrowth will be carried out in-vitro. Conduit materials and designs that do not meet requirements will not be advanced through to in-vivo studies.

Our human tissue work (described above) gives us considerable insight into the mechanisms underpinning neuropathic pain. These tissues allow us to identify molecules whose expression is linked with presence and severity of neuropathic pain, so reduce the number of animals used in our neuropathic pain studies.

At the end of an experiment, we harvest as many tissues as possible post-mortem. If not needed for immediate analysis the tissues are frozen them and are then available for our own future research or that of other researchers working on similar questions.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The nerve injury models intended to be used are well established and reproducible, and their associated adverse effects are well known. Anaesthetics will be administered during any surgical work and analgesics will be administered immediately post operatively and subsequently during the recovery period if needed. If unexpected effects become apparent, veterinary advice will be sought and followed immediately, with animals culled if deemed necessary.

Preliminary work assessing the biocompatibility of some nerve conduits has been conducted in vitro and further in vitro assessment will be carried out in the event of changes to either the composition of conduit materials, the conduit design, or the conduit manufacturing process. In vitro assessment may also be carried out to help determine potential toxicity and appropriate dosage of therapeutic agents that are not well established within the present literature. We believe that these precautions will reduce the potential for unexpected adverse effects to occur as a result of conduit implantation and/or therapeutic treatment.

Why can’t you use animals that are less sentient?

We have no reason to expect any significant species differences in nerve regeneration between rodents and man, but there are differences in regenerative abilities in lower order species such as reptiles. We therefore believe that rodents are the appropriate choice for these studies, which is consistent with the majority of previous investigations.

The majority of the work intended to be performed under this licence can be achieved using mice; however, due to size constraints mice are unsuitable for studies investigating small nerves or long nerve defect. Therefore, we believe that in experiments investigating small nerves, or nerve regeneration over relatively long distances, the choice of the rat is justified.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be regularly monitored for signs of weight loss/dehydration and distress during

these studies. The surgical procedure may cause some post-operative discomfort and animals will be given an analgesic immediately post operatively. Analgesics (administered under veterinary guidance) will be repeated subsequently where necessary, on the basis of clinical judgement, based on observation of behaviour such as reluctance to move, vocalisation when moving or when handled, lack of grooming, or autotomy. Potential complications include infection at the operative site (not observed in our previous studies), local discomfort (mild, typical during the initial 24-48 hours post-operation due to the nature of the surgery, rarely observed after this period in our previous studies), and loss of sutures.
The risk of these will be minimised by carrying out surgical procedures in accordance with the principles set out in the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017).

Animals will be inspected on a daily basis and in the event of post-operative complications, animals will be killed unless, in the opinion of the Named Veterinary Surgeon, such complications can be remedied promptly and successfully using no more than minor interventions. If sutures are lost attempts will be made to reclose wounds using cyanoacrylate glue, whilst animals are conscious or animals will be briefly re-anaesthetised in order for wounds to be closed. Animals that show evidence of persistent pain or discomfort (such as loss of weight, loss of body condition and loss of exploratory behaviour), or which lose more than 15% of their body weight, will be humanely killed.

As described above nerve injury can cause a condition known as neuropathic pain. The signs of this condition depend on the particular nerve that has been injured. Injuries to the sciatic nerve (which supplies the leg and foot) can produce a behaviour where the animals bite their toe nails, in some cases this can be more severe and biting may extend to include other parts of the foot. This may be an indicator of neuropathic pain. We very rarely see this behaviour in mice, but occasionally see it in rats. Any animals who develop biting that extends beyond the toe nails will be humanely killed. Pain will be assessed using observations of behaviours as described above (i.e. reluctance to move, vocalisation when moving or when handled, lack of grooming, or autotomy), and via the use of the grimace scale.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the published guidance provided by the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery, the PREPARE guidelines and other guidance from the 3Rs resource library.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly check information on NC3Rs website, we’ve signed up to the NC3Rs newsletter, and attend Regional 3Rs symposia.

78. Physiological regulation of innate immune responses

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

hypoxia, nutrient, inflammation, neutrophil, innate immunity

Animal types	Life stages
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to investigate whether lack of oxygen (hypoxia) and access to nutrients can regulate the ability of white blood cells to generate energy, protect themselves from damaging stresses, kill bacteria, reprogram immune responses, and enable an effective immune response. In the longer term we hope these insights will allow development of novel treatments for diseases of disordered inflammation currently lacking in clinical practice today.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Respiratory disease kills one in five people in the UK, with almost 30,000 of these deaths a consequence of chronic obstructive pulmonary disease (COPD). White blood cells are critical for our defences against infections, but if they remain in the tissues, they cause damage including in diseases such as COPD. Cells of the early (innate) immune response including neutrophils and macrophages are thought to be the major contributors to this damage. To date there remains very little in the way of effective treatment strategies to target this disordered inflammation. One challenge is to design anti- inflammatory therapies that preserve the capacity of the white cells to fight infection. Consequently, inflammatory lung disease remains a significant disease burden to society. If we were able to shed light on some of the basic molecular pathways regulating the innate immune response and identify molecules that can selectively regulate neutrophil production, death, clearance and long term reprogramming, whilst preserving key anti-bacterial functions, this may be of help to the future development of effective anti-inflammatory strategies so desperately needed for the effective treatment for these common and disabling inflammatory lung diseases.

What outputs do you think you will see at the end of this project?

Academic success will be measured annually through presentation of research work within an international forum. Publication of work within high impact biomedical research journals will be used as a further benchmark of success, and the impact these manuscripts have on the broader research community. Parts of this work will generate large datasets which will be freely available to other researchers interested in lung disease and inflammation biology through online data repositories.
Economic impact will be determined indirectly through the number of institutions with whom I develop new or expand existing professional relationships, the development of partnerships with industry, and the knowledge transfer that occurs as a consequence of these interactions. Through the exposure of sixth form students to a week of work experience and BMedSci or MBB students to short 2-to-6-month research projects within our group I also hope to engage individuals who may not pursue an academic career and increase their understanding of the importance of basic science research and what it entails. We further hope to expand our societal engagement with a regular contribution to the science in schools' programme. Through the provision of biomedical research opportunities to postgraduate students, clinical lecturers, clinical fellows, and academic trainees I also aim to develop the academic respiratory physicians of the future.

Who or what will benefit from these outputs, and how?

Scientific community – the discoveries which this programme of work will generate will be presented at national and international meetings and will be published in peer reviewed journals. Large datasets will be freely accessible to other researcher from the field.

Patients and clinicians – our ultimate goal is to develop new therapeutic targets to treat the common and debilitating lung diseases affecting 1 in 5 people in the UK.

How will you look to maximise the outputs of this work?

Attendance at international scientific meetings and presentation of research findings will provide free dissemination of research to other users with a common research interest. The extension of collaborative interests and the contribution to the publication of research articles in high quality biomedical journals will maximise the outputs from this work.

Species and numbers of animals expected to be used

- Mice: 31,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We study mice because their immune system and lung anatomy is similar to human. Well characterised models of infection and inflammation are available in mice and genetically modified mice are available to test key factors controlling the immune response. This is of particular importance when studying neutrophils which are more challenging to genetically alter in the laboratory. We test key hypotheses in human cells in vitro before studying mice to reduce numbers. We study mice from 4 weeks of age to enable access to sufficient numbers of myeloid cells for phenotypic and functional assessment.

Typically, what will be done to an animal used in your project?

Our principal models involve activation of the innate immune response, for example by inhalation or injection of bacterial products or particulates. They may also be subject to changes in oxygen availability (hypoxia), changes in glucose availability, and drugs that directly alter the immune system. We will study physiological, biological and cellular responses. Animals with specific genetic changes to signalling pathways that are important in controlling the innate immune response will be studied in addition to animals that do not have specific genetic alterations. Typically, animals will be exposed to an infection or inflammatory challenge in the tissues including lung, skin and peritoneum, or systemically following the administration of intravenous agents. Acute responses will be studied over 24-48 hours whilst more sustained responses will be studied over 7-28 days to up to 6 months. The majority of animals will be exposed to either one or two concurrent interventions that activate the immune response e.g. exposure to hypoxia and a bacterial product and one additional intervention to augment this response e.g. treatment with a drug to limit the immune response or the study of an animal with a genetic alteration. To explore the importance of oxygen and nutrient availability for inflammation outcomes, animals will be exposed to different levels of environmental oxygenation and alterations in circulating glucose in a chemically induced model of diabetes. Agents will be administered by injection, inhalation, or oral administration. Outcome measures will include physiological assessments for example non-invasive temperature and blood pressure measures, cellular and nutrient changes in blood parameters following blood sampling. These procedures will require the restraint of animals during the sampling process. In a minority of animals (less than 5%) organ function will be ascertained by whole animal imaging in the anaesthetised state, and invasive physiological monitoring undertaken with the use of surgically implanted telemetry devices.

This work will provide fundamental answers as to how the immune system is regulated in a physiological setting and provide new insights into how we can therapeutically target a dysfunctional immune response to improve outcomes for inflammatory disease states for

which no effective treatments currently exist.

What are the expected impacts and/or adverse effects for the animals during your project?

The breeding and maintenance of genetically modified mice will require offspring to be marked and tissue samples taken for genotyping for example by ear clipping and microchipping. Animals used in experimental models will, for the majority, experience immune cell activation, which can result in a diminished appetite, weight loss, subdued/reduced activity, unkempt, mild or moderate piloerection, increased rate of breathing and a drop in body temperature. Adverse effects will be minimised by accurate dosing of agents, by following appropriate anaesthetic protocols and by regular monitoring of mice for evidence of excessive sickness. Activation of the innate immune response within the tissues may result in impaired tissue function leading to increased respiratory effort, local skin inflammation with abscess formation and systemic illness responses as outlined above. Animals experiencing exaggerated sickness responses including sustained respiratory distress, weight loss of 20% or other signs of illness (subdue activity, unkempt, marked piloerection, immobility, pale feet) will be removed from the experiment and killed by a schedule 1 method.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

One quarter of the experimental animals will experience mild severity and three quarters will experience moderate severity. Animals used for breeding and maintenance will fall mainly within a mild severity category.

What will happen to animals used in this project?

- Used in other projects
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The ex vivo study of human peripheral blood cells cannot replicate the complexity of cell-cell interactions and long-term reprogramming of human innate immune responses and their role in host pathogen responses. Whilst we maximise the use of initial in vitro screens, the need to study these interactions in a physiological context in in vivo models remains. Furthermore, neutrophil experimentation remains limited by the inability to genetically modify and age neutrophils in vitro, severely limiting the ability to verify in vitro novel therapeutic targets without the use of genetically modified mice.

Which non-animal alternatives did you consider for use in this project?

The proposed research project would run in parallel to a series of experiments in which we phenotype murine-derived neutrophil cell lines and ex vivo human blood and tissue neutrophils in health and disease states under a range of physiological and pathological culture conditions.

We have also considered the use of non-mammalian animal models.

Why were they not suitable?

The ex vivo study of human cells cannot replicate the complexity of cell-cell communication, or the inflammatory niche. Therefore, whilst we maximise the use of initial in vitro screens, the need to study these biological responses in a physiological context in in vivo models remains. Neutrophil experimentation remains limited by the inability to genetically modify neutrophils in vitro, severely limiting the ability to verify the biological consequence of manipulation of the key signalling pathways without the use of genetically modified mice. Although the use of murine-derived cell lines can help in this regard and may enable a reduction in the number of mice needed, the testing of the in vivo consequences and the inflammatory niche aspects still require the use of animal experimentation. Furthermore, understanding central reprogramming of neutrophil production and the consequences for tissue effector function requires the study of neutrophils in an in vivo state.

Non-mammalian models are not currently suitable models because of major differences in pulmonary anatomy and in immunological systems. Where advances allow we will incorporate their use to reduce mammalian studies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We calculated numbers of mice based on previous published data from our group and others. We have employed standard statistical methodologies to understand how many animals will be needed to generate robust data. We've used our annual return of procedures data to estimate the number of animals that we will need to use for breeding.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We employed the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs') experimental design guidance and experimental design assistant (EDA) to ensure appropriate numbers of animals are used.

We minimise numbers of mice by collecting the maximum sample amounts and types from individual mice. We use the most up-to-date techniques to reduce the requirement for cell numbers where possible and closely examine published literature in the field to avoid duplication of experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Before each experiment is conducted, a detailed protocol will be written covering (i) a statement of the experimental objectives; (ii) a description of the experiment, covering such matters as the experimental treatments, the size of the experiment, and the experimental material; and (iii) an outline of the method of analysis of the results. Factorial designs are preferred, and power analysis is used where appropriate. Pilot studies will be used where appropriate to generate preliminary data whilst minimizing the size of the experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse is the species of choice for three reasons: First, there is extensive experience in studying immune biology in murine models in our laboratory and others. Secondly, the transgenic models we propose to study are largely confined to the mouse and central to this proposal. Thirdly, to understand central reprogramming of peripheral neutrophil effector function requires access to bone marrow, blood and tissue neutrophil compartments. In the context of inflammatory responses, outcomes are dependent upon the route of delivery, the dose of pathogen/pathogenic product or sterile agent, the strain of pathogen/pathogenic product and the genetic background of the mice. We have already extensively performed dose titrations on all the agents and so we do not anticipate significant morbidity or mortality. For the viral models, we have titrated the doses of virus down to induce only very mild clinical symptoms and typically <10% weight loss when used as a single insult. Where multiple administrations of inflammatory insults occur, mice may theoretically be more susceptible to the effects of pulmonary inflammation/infection after sequential inflammatory mediators (e.g. repeated bacterial challenge). To minimise the potential for adverse effects these experiments will only proceed using doses of mediators which, on their own, would be expected to cause minimal clinical signs of mild piloerection, <10% weight loss, no reduced mobility and normal respiratory rate. This is specifically designed such that the combined stimuli are expected to produce a mild-moderate severity banding.

Why can't you use animals that are less sentient?

These proposed studies require organisms with fully developed respiratory and innate

immune systems.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

As a research group with more than 10 years of experience in modelling the innate immune response, we have developed a general physiological scoring system that we use to monitor the animals on an experimental procedure. Prior to a new program of experiments a discussion with the institute's veterinary team will take place in order to decide appropriate frequency of monitoring of animals based on the expected signs for each agent used. In some instances, supportive measures such as supplemental oxygen will be given to reduce the severity of any adverse effects. Animals displaying 20% weight loss, inappetence, unkempt, reduced activity, marked piloerection, immobility or pale feet will be removed from the experiment and killed by a schedule 1 method. We have specifically included the use of an external rectal thermometer to measure core body temperature to allow our results to be directly compared with previous studies. I have also added the potential to measure temperature by less invasive infrared thermometer to give us the opportunity to directly compare both methods and in future refine our studies.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We have consulted the NC3Rs and the ARRIVE guidelines to ensure both that experiments are conducted in the most refined way and that our work is subsequently reported in a way which maximises published information and minimises unnecessary studies. Study of the PREPARE guidelines and review of the FRAME websites prior to planning new experimental programs enables us to consider factors that are not readily available in the scientific literature which can influence the validity and outcome of studies on animals, improving our overall experimental design.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

To stay informed about advances in the 3Rs, members of my research group and I will attend local meetings targeted at reviewing the 3Rs and understanding animal research. We follow this up within my own research group with quarterly meetings every year to revisit animal research within the group to enable effective implementation of the 3Rs.

79. Development of research grade reagent antibodies for commercial use

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Antibodies, Antigen, Disease, Monoclonal, Biomedical

Animal types	Life stages
Rabbits	Juvenile, Adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to generate novel Rabbit Monoclonal antibodies, which will be available commercially for Research Use Only (RUO). Antibody discovery will also be offered as a commercial service for research.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Monoclonal antibodies are proteins, which bind with high specificity to a target molecule. This unique specificity and ability to detect other biomolecules makes them a crucial

reagent for life scientists undertaking scientific research and for understanding disease, with the potential for developing new therapeutic treatments from such research.

There remains an ongoing need for this type of reagent, to be developed for scientific research. Developing these antibodies will bridge the gap in research areas where key antibodies are either a) missing or are not yet available, b) not of appropriate quality or c) not working in the key scientific applications critical to scientific study.

Monoclonal antibodies or binding reagents (such as aptamers or affimers) can also be generated by non-animal based methods (in vitro methods), with the use of techniques such as phage, yeast, ribosome, mammalian display or in -vitro evolution. These techniques rely on creating a ‘library’ of potential binding regions, which are then screened against the target molecule of interest.

For these projects and in the absence of any pre-existing antibodies which meet the required specifications, the use of animal-derived (in vivo) methodologies is recommended, as the antibodies generated by these methods still outperform those generated with the use non-animal-based systems for the following reasons:

- The immunological processes of animals constantly refines antibodies over time to improve affinity (strength of binding). Although in vitro methods can also be used to refine the affinity of antibodies it is not always guaranteed.
- Antibodies that are generated through in-vivo processes are highly specific to the target (due to exposure to multiple molecules in the blood and immunological tissues of the animal).
- In-vivo derived antibodies are naturally stable molecules, and have been selected by the animal to be so. They have less propensity to aggregate (stick-together) or fragment over time.

In addition, immunisation can better accommodate a wider range of target immunogen types (such as cells, DNA, RNA or cell membranes), than non-animal techniques which rely heavily on proteins and peptides.

When considering different animals as hosts for immunisation, Rabbits as a species, have unique immune responses to target molecules which makes them particularly suited to this work.

- Rabbit Antibodies are particularly stable (even more so than their rodent counterparts, due to slight changes in their structure).
- Rabbits have an additional mechanism for producing a wide range of different antibodies called somatic gene conversion, in addition to the somatic hypermutation present in all rodent species.
- Rabbits produce very high affinity antibodies, and these are particularly well suited as binding reagents.

What outputs do you think you will see at the end of this project?

The development of Monoclonal antibodies will extend across multiple research areas, including but not limited to Immuno-oncology, Oncology, Epigenetics, Neuroscience, Metabolism and Infectious diseases. These biomedical research areas have multiple requirements for high quality, stable research-grade monoclonal antibodies.

The production and validation of multiple new antibodies developed for RUO purposes will serve as a toolbox for the research community to deliver its research goals. The strategy is to bridge the gap in biomedical research areas where key antibodies are:

- missing (not developed, target is novel) or
- not of appropriate quality (specificity is an issue, poorly characterised and validated) or
- not working (or meeting the requirements of) the laboratories' applications, which are critical to studying functionality, specific pathways and diseases e.g. Immunohistochemistry (IHC), Immunocytochemistry (ICC), Flow cytometry (FC), Western Blot (WB), etc.

The novel antibodies to be generated, aim to address such gaps, for the benefit of life science researchers. It is anticipated that the data generated will lay the foundation for pre-clinical studies to clinical trials potentially adding therapeutic value from bench to bedside.

Who or what will benefit from these outputs, and how?

Research scientists will have access to the new antibodies to study disease effects, outcomes and pathways. Thus enhancing our understanding of common and rare diseases.

- The short-term benefit following the launch of RUO products will be for research scientists; to provide them with antibodies that are not commercially available for their research requirements.
- The medium-term benefit will be for commercial companies, research and development (and preclinical) programs within pharmaceutical companies, for various diseases.
- In the long- term it is anticipated that the benefits will be for patients and the clinicians treating them through improved diagnostics and treatments devised using the novel antibody research tools.

How will you look to maximise the outputs of this work?

Our antibody development process is built around the generation of recombinant antibodies – antibodies where the DNA sequences encoding the antibody heavy and light chains are defined and stored within our databases to support future applications.

- This supports long term reliable and reproducible antibody production, which has extended value. By defining the sequence we produce a reliable and defined product which is unaffected by batch variation and more reproducible than polyclonal products.
- We can use this sequence to extend the use of the antibody product into other areas of product development like conjugates, kits, Fc switched products etc to support the needs our research community. This conversion can be performed easily by molecular biology techniques and does not require the use of any further animals to generate.
- Sequences of our sister clones (alternative clones against a single target) are also stored within our database. This supports the development of antibody pairs for kits and also provides alternative products for customers to try in the event that clones advertised do not work on their unique platforms.
- This sequence database is also now feeding into the development of our AI platforms, with the ultimate aim of creating antibodies *in silico* without the need for further animal experimentation.

In addition to our sequencing approaches, we advocate for collaboration and are constantly supporting researchers with new knowledge flowing both ways. Lessons learnt from unsuccessful projects will provide data driven strategies for future antibody development projects.

Species and numbers of animals expected to be used

- Rabbits: 1020

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

- Unique recognition of small molecules and chemicals.

Rabbits are responsive (and generate antibodies) to small molecules and chemicals such as carbohydrates and small-molecule drugs. These types of molecules only produce weak responses in other rodent species.

- Stability/storage properties

Rabbit antibodies are very stable as a product, as they contain slight structural differences to antibodies derived from other species.

- Increased diversity

Rabbit differs from mouse and rat in its immunological mechanisms for antibody generation. Rabbits have an additional mechanism for producing antibody diversity - Somatic gene conversion, in addition to the somatic hypermutation present in all rodent species. This directly increases the diversity of the antibody repertoire, enabling sampling of a wider number of sequences generated in response to antigens.

- Less similarity between rabbit and human proteins

There is greater evolutionary distance between rabbits and humans, so there is less similarity between proteins from these two species. As a result, rabbits may produce antibodies against human protein targets that would be ignored by the mouse immune system.

We use animals for the depth of the antibody response and the subtle differences provided by the affinity maturation response (is the process whereby the immune system generates antibodies of higher affinities during a response to antigen). Some of these subtle variations may result in differences in affinity, application performance and epitopes, which may be the difference between a best-selling antibody (with utility for multiple applications) over an antibody which only works in a single application or is poorly sensitive.

We plan to use rabbits in their juvenile life stage (16 - 20 weeks) once their immune system is developed and keep them into their adult life stage.

Typically, what will be done to an animal used in your project?

Protocol 1 -

Naive animals (protocol 1) will be terminally anesthetised without recovery, a blood sample will be taken by cardiac puncture and the animal humanely killed followed by a secondary confirmation of death.

Protocol 2 -

Prior to immunisation an initial blood sample will be taken intravenously from the marginal ear vein / artery (AB L) to determine baseline blood antibody level titre (a quantitative measurement used to determine the concentration of specific antibodies levels).

Following this, the animal will be dosed subcutaneously (under the skin) with a substance such as antigen, protein or bacteria in combination with an adjuvant (enhance the cell-mediated immune response to the antigen) such as, but not limited to, Freund's Complete Adjuvant ((FCA) is a solution of antigen emulsified in mineral oil and used as an immunopotentiator (booster). FCA is composed of inactivated and dried mycobacteria). Due to the nature of the FCA it will be administered subcutaneously only on one occasion.

Typically, 3 boost immunisations (depending on immunisation protocol length and response in blood titres) with adjuvant such as Freund's Incomplete adjuvant ((FIA) lacks the mycobacterial components (hence just the water in oil emulsion)) will be administered subcutaneously (under the skin).

This schedule will be conducted over a period of up to 110 days with a maximum of 7 immunisations (including prime) and 7 bleeds.

Prior to each boost immunisation, the animal will have a blood sample taken intravenously from the marginal ear vein / artery (AB L) to determine the blood antibody titre levels.

A final pre-splenectomy boost without adjuvant may be delivered intravenously (into or by the vein) (AB L)

At study end, under terminal anaesthesia without recovery whole blood will be collected by cardiac puncture. After humane killing by overdose of anaesthetic and secondary confirmation of death, tissues such as spleen, lymph nodes and bone marrow will be collected to maximise output from antibody development study.

Animals that fail to respond within four 'boosts' will not be subjected to further boosts, the project will be terminated and tissues (whole blood, spleen, lymph nodes and bone marrow) will be collected from the animals and proceed with our in-house hybridoma technology antibody development platforms to identify leads. Part of our assessment on bleed titre includes the evaluation of IgM vs IgG levels enabling us to make evidence-based decisions on which antibody development platforms to consider, especially in cases where responses within 4 boosts are negligible.

A partial response would warrant an additional boost.

We advocate the 3Rs and post collection of materials required by us for the purposes of antibody development, any tissues that can be shared for research training purposes within the remit of the licence is acceptable.

What are the expected impacts and/or adverse effects for the animals during your project?

Following immunisation most animals will not experience any adverse effects greater than mild discomfort from the injection.

Injection site reactions

An estimated 1% of animals may develop non-septic granulomas (is an aggregation of macrophages (along with other cells) that forms in response to chronic inflammation. This occurs when the immune system attempts to isolate foreign substances that it is otherwise unable to eliminate). The presence of granulomas may not resolve quickly and may persist throughout the immunisation protocol. These are more likely to be seen on later immunisations once there is a higher titre of matured antibodies present. Immunisation campaigns will be initiated with the mildest regime and adjuvants possible.

Typically, the granuloma will be less than 1 cm in diameter and should not cause any pain or discomfort. Animals with granulomas will be humanely killed if the granulomas appear to cause irritation or impair movement or the granulomas are not free moving with the skin (no invasion of the muscle layer).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

100% of animals on Protocol 1 will only experience non recovery.

100% of animals on Protocol 2 are expected to be mild severity.

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

1) Alternative platforms for antibody discovery, although used for therapeutic antibody generation are not suitable for the development of reagent-grade (RUO) antibodies for the following reasons:

- Alternative platforms such as phage/yeast or other display technologies perform poorly against linear or complex targets (such as cell surface receptors).
- Affinity (strength of binding) from these platforms is generally poor. This is not suitable for targets which have very low expression in tissues or cells, as they will fail to detect the protein. Affinity maturation techniques are not guaranteed to improve the binding in these scenarios.

- Natively paired antibodies (such as the ones produced by animals) are more stable and produce higher yields than non-natively produced antibodies.

We are currently developing methods for animal replacement, which are not yet implemented. To support the use of non-animal derived methods, we sequence all antibody genes produced in our processes. These sequences are stored securely for use not only for recombinant production or synthesis, but also for the purpose of training AI models which we can use to support project development in the future.

We will continue to explore the use of third parties/collaborations to utilise technologies which are currently outside our capabilities.

Which non-animal alternatives did you consider for use in this project?

A number of animal-free methods, based on a technology known as phage display and Artificial Intelligence/Machine Learning have been trialled, to determine if such techniques are suitable for discovering new antibodies for research use. These techniques have been tested in-house and also with 3rd party suppliers/vendors.

Why were they not suitable?

We have trialled multiple non-animal (alternative) methods for producing antibodies both through internal research and development (R &D) projects, and using external suppliers with multiple projects attempted using phage display. These have yielded very low success rates in terms of antibodies relevant to researchers, low quality and lack of specificity.

These findings have led to further development of in-house R &D using methodology that does not completely eliminate the use of animals but which can reduce the numbers. We continue to develop non-animal alternatives, and will implement these when we have strong evidence that the antibodies developed by these methods are a suitable alternative to the ones developed by animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

In situations where antibodies are available commercially from other organisations, we make every effort to in-license or internalise pre-existing products from external manufacturers, thereby further reducing the use of animals.

As a commercial business we initiate more than 100 new Rabbit Monoclonal antibody projects or targets per year to ensure reagents for newly-discovered research targets are available to the life sciences research community. This is to ensure coverage of applications (such as IHC/ICC/Flow cytometry etc) that research scientists may require for each of their targets, to maintain a breadth of targets on the catalogue, and to drive revenue for the business.

The prospective target list is driven by real-time data derived from research publications, collaborations with academic institutions, public-domain information, search requests and conference attendance. It is worth highlighting that only targets which are required by the research community are developed. The refinements to methodologies made to date, have ensured that typically 2 rabbits are used per project/target; the 100 targets required by the business equate to 200 rabbits per year across the span of the project licence. As antibody serum titre/immunogen response in each rabbit is not guaranteed (despite the use of multiple tools to help increase predictive immunogenicity) using two rabbits mitigates the risk of non-response by up to 50% and therefore this is critical to ensure targets are given the best chance of success and prevent any repetition and further use of rabbits. This is the rationale for using 200, rather than 100 rabbits for this process.

Once non-animal alternative platforms are available, we predict that the numbers of rabbits to drop over time, as we route projects (where possible) through suitable alternative platforms.

We are using multiple tissues (whole blood, lymph nodes, bone marrow, spleens) to maximise the options for discovery.

We cryopreserve all excess materials for future discovery projects to reduce animal usage.

Rabbits have larger spleens and other immune organs than mice, which produces more lymphocytes (B cells) which are then available for producing antibodies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have reduced the number of rabbits to 2 per project and replace animals where possible. We always use the fewest number of animals possible for each study and will undertake additional measures to limit animal numbers – e.g. using proprietary peptide design strategies, informed by years of experience using rabbits to generate recombinant antibodies, multiplexing immunogens, if appropriate, ensuring immunogens are high quality. Cryogenic storage of tissues from a project ensures opportunity for further investigations and repurposing where required.

We will conduct a case-by-case evaluation of each project in association with the AWERB committee and all options concerning the reduction in the number of animals will be prioritised. Wherever opportunities exist to improve our commitment to the 3Rs, this will be communicated and diligently implemented. Experimental designs have reduced the number of rabbits used per project for antibody development. This has been achieved through constant improvements in our development methods. We are also committed to constantly innovating and evolving our R&D on animal free platforms involving phage display and AI implementation. Although animal-free platforms are in their infancy, we are committed to using/trialling them wherever possible, in order to replace animals with animal-free methods for antibody development.

Cryopreservation on critical animal materials enables us to repurpose and rescue projects where required without further immunisations and animal usage. Sequencing the monoclonal antibodies ensure long term access to reproducible antibodies without the need for further immunisations in rabbits.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have reduced the number of rabbits used per project for antibody development. This has been achieved through constant improvements in our development methods.

The following improvements have enabled us to optimise reduction of animals used:

a) Use and validation of immunisation using multiple peptides, to generate antibodies against multiple epitopes within the same animal, to reduce animal numbers.
b) Improvement and development in antibody discovery platforms (such as single B-cell cloning and improvements in B-cell enrichment and hybridoma generation), increases our success rates for developing successful antibody panels, reducing requirements for alternative strategies or repeat immunisations.
c) Creation of recombinant multiclonal products (mixtures of monoclonal antibodies) to replace traditional polyclonal products.
d) Evaluation of targets for suitability as hybrid projects, to co-develop multiple products from the same immunisation.
e) Up validating pre-existing recombinant antibodies for performance in previously untested applications, means we can serve customer requirements without use of additional animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Animal studies are designed to minimise pain and distress.

We have actively worked towards refining our animal procedures and have eliminated ascites production as well as removal from sale any products generated using the method.

We actively ensure none of our projects exceed 110 days of immunisation protocol length.

We often use adjuvants such as Freunds complete adjuvant (FCA) for the first immunisation only, with follow-up boosts using adjuvant such as Freunds incomplete adjuvant (FIA). The volume of injection is limited to 1ml (50:50 Antigen: Adjuvant) administered over 4 sites of injection. This immunisation process has resulted in high success rates for delivering both internal and customer projects.

Adjuvant used will be matched to a specific antigen. Adjuvant used may be, but not limited to, either water in oil adjuvant (FCA/FIA) or oil in water adjuvant (Ribi).

The animals will be group housed in floor pens within stable, compatible groups. The animals will have access to enrichment such as hay for foraging, play, nest building, and

for a varied diet. Gnawing material will be provided to prevent the teeth from overgrowing. Substitute burrows will be provided for retreat in fear-provoking situations and to manage social interactions. Raised areas will be used to make use of the vertical space, offer a comfortable resting place and refuge, stimulate exercise and offer a choice of microenvironment.

Animals will be given gentle and frequent handling and be habituated to procedures.

A topical anaesthetic will be applied to the marginal ear vein / artery (AB L) prior to blood sampling to cause the least pain, suffering, distress or lasting harm to the animals.

Why can’t you use animals that are less sentient?

Worms, flies, fish, toads, etc are not appropriate species to use in this project because the information is not translatable and the work needs to be performed in mammals.

Our rationale for using rabbits is that the rabbit differs from mouse and rat in its immunological mechanisms for antibody generation. This leads to a diverse antibody repertoire and maximizes opportunities to develop antibodies with key properties required for addressing project needs enabling the advancement of research to address unmet needs.

Rabbit antibodies have a number of advantages over traditional mouse antibodies.

Rabbit IgG is more suitable as a commercial product as it contains an additional disulphide bond which makes the proteins more stable than their rodent counterparts. In addition, rabbits only make a single isotype of IgG (compared to 4 isotypes in mouse/rat). These factors affect both the utility (ease to use in a commercial process) and the stability of the commercial product.

Rabbit differs from mouse and rat in its immunological mechanisms for antibody generation. Rabbits have an additional mechanism for producing antibody diversity - Somatic gene conversion, in addition to the somatic hypermutation present in all rodent species. This directly increases the diversity of the antibody repertoire, enabling sampling of a wider number of sequences generated in response to antigens.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We are driven to constantly evolve and make data driven improvements to our methodologies.

Animals will be housed together in floor pens throughout the duration of the protocol with continuous access to food, water and suitable enrichment. Prior to any blood withdrawal via the marginal ear vein / artery a topical anaesthetic such as EMLA will be applied. Animals will be immediately monitored post blood withdrawal and / or post immunisation and checked again at the end of the day. The animals will be checked every day where / if necessary and the frequency of the monitoring may be increased should it be required. If problems arise we can act quickly to prevent further problems. The Named Veterinary Surgeon (NVS) will be consulted if required.

We give consideration and review the most appropriate adjuvants to be used.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

If and when appropriate, the Experimental design assistance (EDA) developed by the NC3Rs will also be utilised to help validate study design and also to enhance understanding of overall study design by providing useful visualisations of experimental groups and commissions.

The NC3Rs good practice guidance documents will also be utilised when performing administrations and blood withdrawals.

Where appropriate we will act in best accordance with the ARRIVE (2.0) and PREPARE guidelines from the Secretary of Norecopa/RSPCA particularly with regard to the equivalent methods sections.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will conduct a case by case evaluation of each project in association with AWERB committee and all options considering the welfare of animals will be prioritised. Wherever opportunities exist to improve our commitment to the 3Rs , this will be communicated and diligently implemented, principally by working with the AWERB.

Also, by subscription to regular newsletters from groups such as the NC3Rs, LASA and the RSPCA as well as attending relevant courses and conferences will help to stay informed on best practice. Close liaison with the Named Information Officer at the establishment will also facilitate efficient passage of information.

The NC3Rs 3Rs resource library will also be referred to during the project to help maintain best practice as well as frequent discussions with our in-house compliance team.

80. Kin recognition mechanisms and the fitness consequences of discrimination rules

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

kin recognition, kin selection, discrimination, cooperation, inbreeding

Animal types	Life stages
long-tailed tit	Neonate, Juvenile, Adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to determine the mechanism(s) used by a social bird to recognise relatives and the accuracy of information conveyed about relatedness. To achieve this aim we need to use molecular genotyping to determine the genetic relatedness of individuals living in a wild population of the study species, the long-tailed tit *Aegithalos caudatus*.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The functioning of biological systems at every level of organisation from genes to complex societies requires effective systems of recognition. For example, immune systems must discriminate between self and non-self, and in social organisms individuals must discriminate between desirable and undesirable social partners. Therefore, natural selection is expected to have engineered effective recognition mechanisms. However, recognition systems are rarely error-free and a substantial body of theory has been developed to understand how the frequency of errors is optimised. The aim of this study is

to investigate kin recognition mechanisms and their efficacy in a social bird, the long-tailed tit *Aegithalos caudatus*, whose remarkable breeding system makes them ideally suited to investigation of kin discrimination and its consequences. Long-tailed tits discriminate against close kin as mates and in favour of kin when cooperating, but they make frequent, apparently maladaptive errors in both contexts. We will employ a range of state-of-the-art methods to investigate three mechanisms of kin recognition: familiarity, vocal cues, and olfactory cues, determining how accurately they encode relatedness information. We will then determine the extent to which these modes of recognition are integrated to optimise behavioural decisions. The outcome will be a major advance in understanding of how and why good and bad social decisions occur, providing general insights into how mechanisms constrain evolutionary adaptation.

What outputs do you think you will see at the end of this project?

The principal outputs from this study will be scientific papers that describe new findings about recognition systems and social evolution, published in leading international journals. Those findings will also be communicated to non-scientific audiences to improve public understanding of the study system and of evolutionary processes.

Who or what will benefit from these outputs, and how?

The principal beneficiaries will be evolutionary and behavioural scientists interested in recognition mechanisms and social evolution. The project will be testing fundamental theory on signalling and communication so it will have broad relevance to anyone interested in recognition systems at any level of biological organisation. The project will also involve the development of AI machine-learning tools for classification of signals that will be of widespread interest in this rapidly growing field. Outputs are expected to be published from 2025 onwards.

How will you look to maximise the outputs of this work?

Members of the research team will publish papers in high-ranking international journals, and give talks at national and international conferences. The PI and other members of the research team are also committed to public outreach and will seek opportunities to publicise the project to enhance public understanding of science.

Species and numbers of animals expected to be used

- Other birds:
    - long-tailed tit: 1250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The long-tailed tit is a model system for the study of social evolution. This project builds on the findings of a long-term study (initiated by the PI in 1994) of the behaviour and ecology

of a wild population of this species, providing a robust rationale for its objectives. The regulated procedure required (blood sampling by brachial venipuncture) is mild and has no adverse effect on the animals, whose welfare is paramount for the collection of long-term behavioural and life-history data. Blood samples will be taken from nestlings (12 days old), juveniles and adults, according to the age of first capture.

Typically, what will be done to an animal used in your project?

A bird will be captured in a mist-net (adult or juvenile), extracted and placed alone in a clean cotton bag; or, taken from a nest (nestling) and placed in a clean cotton bag with other members of its brood. All birds will be held in bags for a maximum of 30 minutes, and in most cases <10 minutes. Individuals will be taken from the bag, ringed, biometric data recorded and a sample of preen oil collected from the uropygial gland prior to the regulated procedure. For brachial venipuncture, the bird will be held in the ringer's grip with the left wing extended to expose the brachial vein. The underwing will be swabbed with water and a small puncture made in the brachial vein using a microlance (0.5 x 16mm) to produce a drop of blood. Blood (10-25 microlitres) will be drawn up into a heparinized capillary tube and a small piece of cotton wool will then be held against the underwing to staunch any blood flow. Birds will then be released (or replaced in their nest in the case of nestlings) following assessment of their wellbeing. Total handling time for each bird is typically <5 minutes.

What are the expected impacts and/or adverse effects for the animals during your project?

Mild stress from capture and handling. Mild pain from brachial venipuncture. Any effects are short-term, with no medium- or long-term consequences.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild severity for all sampled individuals.

What will happen to animals used in this project?

- Set free

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The use of wild long-tailed tits in their normal environment is essential to achieve the academic objectives of this project, which is to observe behavioural decisions and the mechanisms underlying them in a natural context.

Which non-animal alternatives did you consider for use in this project?

There is no alternative to the use of animals for testing theories about behavioural decisions relating to mate choice and cooperative behaviour.

Why were they not suitable?

N/A

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The aim is to individually mark and genotype all members of the study population. The study population typically comprises 50-70 breeding pairs with annual production of 100-200 offspring. The maximum number of birds processed in a year is c.250, so over 5 years and given that each bird is sampled only once, it is estimated that a maximum of 1250 birds will undergo the regulated procedure.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

It is essential that as many birds in the study population as possible (typically >95%) are individually marked and genotyped to determine the social environment for all individuals for which behavioural decisions are observed. Therefore, there can be no reduction of animals used because it would result in incomplete knowledge about relatedness among birds whose behaviour we are observing.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The aim is to sample all members of the population, requiring capture effort from March to June and October to November in each year of the project.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why

these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Long-tailed tits will be used for this project. Brachial venipuncture is the only regulated procedure to be used; this is the standard technique for obtaining blood samples from small passerine birds, causing only short-term, mild discomfort. In addition, we will record standard biometric data and fit rings to birds, under British Trust for Ornithology (BTO) licence, and collect a small sample of preen oil from the uropygial gland.

Why can’t you use animals that are less sentient?

The long-tailed tit is a model system for the study of social evolution. This project builds on the findings of long-term study (since 1994) of the behaviour and ecology of this species at the proposed study site, providing a robust rationale for the project's objectives.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The PI works closely with colleagues at a UKRI-funded genomics facility for genotyping. Any development in genotyping techniques that allow for refinement of techniques for DNA-sampling to reduce potential harms to the birds will be implemented. The avoidance of harm to birds through catching, handling and ringing birds is a key component of training for a BTO licence, and compliance with rules set out in the BTO's Ringers' Manual (Redfern & Clark 2001) is required for annual licence renewal. It should also be noted that collection of long-term behavioural and life-history data is essential for success of the project, so the PI and his research team are well placed to monitor any harms resulting from procedures through behavioural observations in the field.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All catching, handling and ringing of birds follows the best practice guidelines stipulated in the BTO's Ringers' Manual (Redfern & Clark 2001).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The PI is a regular reviewer of grant applications and scientific articles submitted to national and international funding agencies and journals. The majority of these concern animal behaviour, and the welfare of study species and compliance with ethical guidelines and regulatory bodies is usually a critical part of the reviewing process. Therefore, I am routinely informed on the ethics and implementation of research by professional bodies and societies to ensure compliance. In addition, I will be informed of advances in the 3Rs via email communications from my own establishment during the course of this project.

81. Incorporating infectious disease exposure screening into improved estimated breeding value (EBV) sheep breeding program modelling.

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Key words

health status, resistance, resilience, sheep

Animal types	Life stages
Sheep	Juvenile, Adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the project is to collect the relevant disease screening data from sheep in a selective breeding program in order to statistically model the influence of the health status on the performance and therefore estimated breeding value (EBV) of animals in the population.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Robustness and efficiency are essential attributes for commercial lamb producing flocks. Robust sheep adapt to the variable weather and management conditions from year to year

and season to season, without becoming ill or requiring human intervention, this is better for the welfare of the sheep. Efficient sheep produce more lambs per ewe per year with less feed input, resulting in a lower carbon footprint and more cost effective product for the producer and consumer.

In order to be able to select for the most robust and efficient individuals in any breeding program it is necessary to account for and thereby exclude from our genetic comparison models, as much of the variation between individuals which is not due directly to the genotype. It is also important to conduct genetic evaluation in an environment and under conditions which are as similar as possible to those in which the selected individuals are expected to be farmed as there are inevitably indirect genotype- environment (GE) interactions which influence the ultimate performance of individuals selected for traits such as growth rate or fertility. Accounting for variations in health status, particularly exposure to infectious pathogens is one important but poorly understood aspect of this GE process. The purpose of this work will be to quantify the influence of infectious disease health status on the growth rate, fertility and morbidity of sheep in a large scale breeding program. This is the first step in a much longer process of identifying both resilient and resistant genotypes of sheep, which is beyond the scope of this current project but which depends upon it as a foundation.

What outputs do you think you will see at the end of this project?

The primary output of this project will be sheep which are better suited to the commercial farming environment by virtue of having been selected using a more appropriate and comprehensive genetic selection algorithm which incorporates the health screening data along with production traits.

The secondary output will be the generalisable selection algorithm which will be published in a peer reviewed scientific journal and thereby disseminate the research results to the wider sheep industry where it can be adapted for other breeding programs.

Who or what will benefit from these outputs, and how?

Over the short term the individual sheep selected from the breeding program will directly benefit from the improved welfare, while the farmers will benefit from the improved performance and economic efficiency of the sheep selected for breeding.

In the long term the compound benefit of successive generations of selection on this improved basis will yield continued improvements in these key areas, leading to dissemination of these genetic benefits to the wider industry. Healthier, more efficient and productive sheep are also more profitable, leading to an economic benefit for the lamb producer, an environmental benefit to society from lower green house gas footprint from our food, and a benefit to the consumer by reducing the cost of lamb meat.

How will you look to maximise the outputs of this work?

The outcomes will be disseminated via scientific publications and presentations at both scientific and agri-livestock conferences.

Species and numbers of animals expected to be used

- Sheep: 15,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Sheep (over 1 year old) will be used because the research objective is specific to sheep in a specific breeding program and >1yr old animals will be sampled because the infectious pathogens in question only manifest in the older animal in a detectable form.

Typically, what will be done to an animal used in your project?

One venous blood sample of 7-10ml will be collected for health screening. This is the only regulated procedure which will be conducted. Measurement of production traits such as growth rate are routine, non-invasive and done in accordance with normal agricultural practice.

What are the expected impacts and/or adverse effects for the animals during your project?

The sheep will experience a brief discomfort while the blood sample is collected which typically takes less than a minute.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Severity will be mild for all animals.

What will happen to animals used in this project?

- Rehomed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The project aims to discover the influence of health status on performance of sheep under the management conditions of the selective breeding program which means sampling the minimum number of sheep in the breeding program by the least invasive method possible is integral to the objective of the project. It is important to do this in naturally exposed sheep in the farm environment so that the findings of the study are relevant to the real

world situation.

Which non-animal alternatives did you consider for use in this project?

There are no non-animal alternatives to this.

Why were they not suitable?

There are no non-animal alternatives to this.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimation is based upon the population size of the breeding program (approx. 20,000 breeding ewes, producing >38,000 lambs per year), the demographic structure (>28 different locations and management groups) and finally the test characteristics (sensitivity and specificity) of the screening tests for diseases of interest which are available. The minimum proportion of animals will be screened per year to establish health status at the management group level.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The proportion of animals required to be tested is calculated by a Bayesian sample size calculator developed specifically for this purpose to estimate the number of animals required to be sampled for a given group size, underlying disease prevalence and in the context of the best available information on the accuracy (sensitivity and specificity) of the laboratory test.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Statistical modelling will be used to infer exposure to a given pathogen for animals within each management group from the screening results of a proportion of each group thus avoiding the need to test every animal and thereby substantially reducing the number of animals needed for the study.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime

of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

To determine the health status of the management groups it is necessary to sample the minimum number of animals to achieve a representative sample of animals from the group. The method proposed (single blood sample) is minimally invasive and is the only method currently available for acquiring the necessary quantity of blood to test for antibodies and antigen and blood is the only material which can be used for the laboratory test.

Why can’t you use animals that are less sentient?

The research objectives are specific to the sheep in this breeding program so it is not possible to use other species.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will collect the minimum number of samples and minimally invasive samples from the sheep. Sampling will be brief (<1minuite per animal). All animals will remain on the farm of origin. No animal will be sampled more than once for the research project. The study will be supervised at all times by an experienced farm animal veterinary surgeon. All staff involved in farm sampling will be experienced in handling animals and trained personal licence holders. Where appropriate, sampling will be linked with handling for other husbandry procedures. If an animal becomes distressed by the handling and/or blood sampling procedure, the procedure will be stopped and the animal returned to the flock.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Blood sampling by experienced personel will have minimal adverse impact on the sheep. 'ARRIVE' guidelines will be used for study design and reporting https://arriveguidelines.org/.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The project licence holder will stay informed about advances in 3R's through engagement with the National Centre for Replacement Reduction and Refinement of Animals in Research Website and through seminars and information disseminated through the research institution where the project licence is held.

82. Respiratory Pharmacology

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Respiratory disease, Asthma, COPD, Lung, Animal models

Animal types	Life stages
Mice	Adult
Rats	Adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To investigate and target mechanisms involved in the respiratory related diseases and to develop therapeutic treatments for respiratory diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Respiratory disease impacts 20% of the population and is the third biggest cause of death in England. There is a significant unmet need for new treatments to cure or improve the management of these diseases worldwide. The annual economic burden of asthma and COPD on the NHS in the UK is estimated as £3 billion and £1.9 billion respectively. In

total, all lung conditions (including lung cancer) directly cost the NHS in the UK £11billion annually.

The data generated during this project will be used to identify potential new treatments in the fight against respiratory diseases.

What outputs do you think you will see at the end of this project?

The ultimate output from this project would be the identification of new therapeutic drugs that can potentially treat respiratory diseases.

Studies performed under this licence will generate essential information to support scientific programmes aimed at identifying and understanding new ways of treating diseases of the respiratory system, or to investigate immunological mechanisms.

We will generate data on the effectiveness of potential new medicine in comparison to other treatments, and determine how the drug might distribute throughout the body. The data will contribute to medicine development programmes to address unmet medical need in a range of disease types, to benefit patient health. We may also share the findings in scientific publications to enhance understanding in this field.

Who or what will benefit from these outputs, and how?

There are a number of communities that will potentially benefit from this work. Firstly, by identifying promising new drug candidates, pharmaceutical companies could benefit by taking these drugs forward for further development and potentially bringing them to market. Therefore, patients could benefit significantly from the development of effective new treatments for respiratory diseases, which could improve their quality of life or even save lives. Secondly, finding improvements in animal disease models could benefit future animal research with constant application of 3Rs in the animal models. Lastly, sharing data through publication will contribute to the scientific community's understanding of respiratory diseases and developing more promising treatments for patients.

How will you look to maximise the outputs of this work?

Where possible, we will share our findings in scientific publications as part of the process of investigating and validating new disease targets and new treatments for disease. This continues an established approach, as demonstrated by previously published work. Additionally, the company also supports the view that publication of unsuccessful approaches ('negative data') is a valuable scientific output from properly conducted research, and this type of data would not be excluded from a publication strategy.

Information generated using this licence is stored in a searchable backed up company database, so that it is always accessible to other internal company researchers. Therefore, data will be recoverable in the future, even after likely project and personnel changes, and the information will be a valuable future resource to reduce the need to repeat and re-establish competency in a field of research.

Species and numbers of animals expected to be used

- Mice: 10000

- Rats: 5000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Rodents, particularly mice and rats, are physiologically similar to humans in many ways, making them an effective model for studying human diseases. Their respiratory system, while not identical, shares many similarities with that of humans, allowing us to extrapolate our findings to potential human applications.

We have chosen to use adult rodents because the adult rodent respiratory and immune system is fully developed and mature, providing a more accurate representation of the disease's impact and potential treatments in humans.

Typically, what will be done to an animal used in your project?

In most cases, an immune response will be initiated in an animal through exposure to an agent such as LPS that engages with the innate immune system (the part of the immune system that produces a quick and general response to an environmental challenge) or will use an agent such as an allergen that will trigger an adaptive immune response (a very specific response that takes time to develop but which then will exist for a very long time). Agent exposure could be via injection or direct to airways of the respiratory system by dosing liquids into the nose or direct into the windpipe whilst an animal is briefly and lightly anaesthetised.

Animals will have samples or measurements taken during experiments (e.g. blood samples taken from veins). Animals will receive potential new medicines, either during or after immune responses are established or changes to the respiratory system have occurred. Such compounds could be dosed via a variety of administration routes (e.g. oral, intravenous or via inhalation) on single or multiple occasions, over days or weeks.

All animals will be humanely killed at the end of each experiment to enable collection of samples.

What are the expected impacts and/or adverse effects for the animals during your project?

Following exposure of mice to some challenge reagent (such as LPS) inducing inflammation, weight loss is expected (up to 20% loss of maximum attained bodyweight). Sometimes additional adverse signs can be noted after different types of treatments, (e.g. injections of agents that activate the immune system). These activities could result in subdued behaviour and raised fur lasting for up to 8 hours. Animals may display mild to moderate abnormal breathing if the reagent is administered directly to the airways lasting up to 5 mins.

Expected severity categories and the proportion of animals in each category, per

species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Approximately 80% of animals (mice, rats) are likely to experience moderate levels of severity. The remaining 20% of animals are likely to experience mild severity.

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Like any other immune disorders, respiratory diseases are complex and cannot be adequately studied in vitro (in a petri dish or test tube) or through computer simulations alone. In order to fully understand the effect of a new drug candidates in a complex physiological setting where multiple immune components and communications between different cell types are involved, animal models provide a powerful tool to investigate the response to these potential treatments. This allows for a more comprehensive understanding of the disease process and the drug's effects, which is critical in the early stages of drug development.

Which non-animal alternatives did you consider for use in this project?

in vitro cell culture, 3D culture, spheroid, organoid and ‘lung-on-chip’ systems

Although these technologies cannot fully replace the use of animals in our research, we aim to utilise the data output in a way that is complementary to the animal data. For example, assays employing human material could be cellular, involving individual cells derived from blood or digested tissue, which can be cultured for testing compounds. Another example is in vitro human and murine cellular air-liquid interface (ALI) systems, which permit limited differentiation of epithelial cells and interaction between different cell types, such as epithelial and immune cells. Results from these assays can guide the optimal design and interpretation of data derived from subsequent in vivo assays.

Furthermore, we continue to investigate the applicability of 3-dimensional 'organoid' culture systems. Epithelial organoids are composed of cells within a matrix that supports development and differentiation within a connective tissue environment, resulting in the appearance of functional phenotypic structures with cilia, and orientation (apical and basolateral surfaces).

Computational models and simulations can be employed to predict the effects of new drugs and comprehend disease progression. These models can be based on data from both in vivo and in vitro studies.

Why were they not suitable?

The respiratory system is highly complex, involving multiple cell types, tissues, and organs all interacting together, which is difficult to replicate with in-vitro systems such as organoid or lung on a chip. In addition, respiratory diseases often involve changes that occur over time and potentially have an impact on the whole-body system. Non-animal models cannot accurately replicate these temporal and systemic aspects of disease. Furthermore, the response to potential treatments can be influenced by many factors, including metabolism, immune response, and other physiological processes, which can be challenging to study in non-animal alternatives.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number takes into account of a typical number of animals used in a study. The number is then multiplied by a number of studies likely to be run in a year. This is again multiplied by the number of years (x5) the licence is active for.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use appropriate group sizes, guided by our statisticians, to ensure that our experiments have sufficient power to detect significant effects with the smallest number of animals. Where appropriate, each animal is served as its own control instead of using separate groups of animals for each experimental condition.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

When working with a new model, we will conduct pilot studies with small numbers of animals to help refine our procedures, estimate the variability of our measurements, and accurately calculate the minimum number of animals needed to achieve the scientific objectives of the study. For some model systems, rather than collect only terminal measurements we will employ serial sampling where we can take samples repeatedly from the same animals at different time points to monitor development of pathology or changes resulting from treatment. This approach will reduce the total numbers of animals required to gather decision making data because each animal will act as its own control, thus reducing readout variability and improving quality of data. Statistical information generated from analysing such data is likely to be more robust compared to data derived from single measurements from many different animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Different models of short and long term immune system sensitisation and challenge, targeting distinct aspects of the immune system will be used to investigate the involvement of those processes in human disease. Some models will use chemicals that trigger the immune system. Sometimes, proteins will be given to animals along with chemicals that boost the immune response (acting as an adjuvant) that together cause the immune system to react. The same protein put later into the airways of the respiratory system would then trigger a localised immunological allergic reaction that can be used to test treatments targeted at aspects of the induced immunological pathway in that system. The project will also use extract of house dust mite which is a commonly detected allergen associated with respiratory diseases such as asthma. Some models will include viral or bacterial micro-organism infection with or without additional challenge.

The animal models used for this programme of work have been chosen and, where feasible, will be developed further to represent the least severe and most effective and robust ways of measuring immunology and inflammatory mechanism changes over both short and longer duration experiments. The durations of experiments will be suited to the mechanism under investigation, and in the majority of cases pain, suffering or distress will be brief and symptoms will be transient. Any airway inflammation or infection changes in these experiments is usually mild and would normally not cause any obvious signs of distress. Body weight loss is known to be a sensitive measure of the severity of inflammation. Changes in body weight will be monitored to assess the models, and also limit the possibility that an animal might develop adverse effects that are more severe than are needed to reach an answer to a scientific question. Each approach described above models distinct aspects of human diseases or processes involved in multiple diseases and are carried out for only as long as required for the relevant conditions to develop and potential new treatments to be assessed.

Why can't you use animals that are less sentient?

While it's important to use the least sentient animals possible in research, it's also crucial to ensure that the outcome from an experiment is valid and can contribute to our understanding of disease or the development of new treatments. Although using less sentient animals than rodents might seem more ethical, there are several reasons why they might not be suitable for respiratory research. Firstly, more complex animals, like rodents, often have physiological systems that are more similar to humans. This makes them more suitable for studying human diseases and testing potential treatments. Less sentient animals, like insects or worms, have significantly different biology, which may not provide accurate or relevant results. In addition, the respiratory system is highly complex and involves multiple cell types, tissues, and organs. Less sentient animals do not have a respiratory system that is complex enough to provide a useful model for investigating immunological pathway relevant to human diseases.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

On the day of dosing, animals will be observed closely, then the frequency of observation will be related to the nature and intensity of any adverse effects observed, but will not be less than twice daily for animals being treated. If the condition of an animal gives cause for concern close observation will continue until clear signs of recovery are evident. If the condition of the animal continues to deteriorate, or in the absence of clear signs of recovery, the suitability of the animal to continue on study will be reviewed and appropriate action taken as necessary. If adverse effects cannot be ameliorated using no more than minor interventions such as providing soft palatable diet, additional warmth (bedding or provision of heat), the animal will be humanely killed. There are specific criteria in place under each protocol to determine if a humane endpoint is reached.

When setting up a new model, conducting pilot studies will provide valuable insights into both expected and unexpected clinical signs that may arise during the main study. The outcomes from these pilot studies will be instrumental in refining the procedures and experimental design while maximising data output and minimising potential harm to the animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The published principles and philosophies behind the PREPARE (2018) and original ARRIVE (2010) guidelines have been incorporated into the sponsoring company's internal project planning standards of care and standard operating procedures. All work carried out under authority of this licence will undergo assessment of the study design during planning stages as part of a peer review process that is based on those guidelines, and will include statistical consultation. Facilities and processes are audited by independent bodies such as AAALAC International which has published guidelines and procedures in The Guide (NRC 2011). Our company wide policy covers the standards of care and the ethical treatment of animals in research, development and testing of all our potential medicines. The following published documents will advise on experimental design, animal welfare and husbandry during the life cycle of this licence:

- Kilkenny C et al (2010). Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. PLoS Biol 8(6).
- Smith A et al (2018). PREPARE: guidelines for planning animal research and testing. Lab Anim; 52(2):135-141.
- Percie du Sert N et al. The ARRIVE guidelines 2019: updated guidelines for reporting animal research. BioRxiv. 2019: 703181.
- National Research Council. (2011). Guide for the Care and Use of Laboratory Animals: Eighth Edition. Washington, DC: The National Academies Press.
- NC3R's - Responsibility in the use of animals in bioscience research: expectations of the major research council and charitable funding bodies (2019).
- Guidance on the operation of the Animals (Scientific Procedures) Act 1986. (Home Office 2014).
- LASA - Guiding principles on good practice for animal welfare and ethical review bodies. (2015)

- Prescott MJ, Lidster K. Improving the quality of science through better animal welfare: the NC3Rs strategy. Lab Animal 46(4):152-156, (2017).
- Review of harm-benefit analysis in the use of animals in research. Report of the Animals in Science Committee Harm-Benefit Analysis Sub-Group chaired by Professor Gail Davies (Nov 2017).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The role of the sponsoring company named information officer (NIO) includes the sharing of animal welfare, best practice and 3Rs related information. The NIO also liaises directly with the company project licence holder network through their own regular meetings, and also raises this type of information and discussion points at the institutional Animal Welfare and Ethical Review Body (AWERB). The licence holder is part of the Animal Research Community (ARC) and is also separately aware of the 3R's related work of the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) and the Royal Society for the Prevention of Cruelty to Animals (RSPCA). 3Rs issues and advances are highlighted, discussed and actions are implemented within the company centrally and effectively via these forums. I have also set up alerts on various scientific databases and journals to notify me of any new research, techniques, or methodologies that could potentially enhance our adherence to the 3Rs principles. By attending relevant conferences to respiratory research, I can use the valuable opportunity to discover new information on 3Rs, and gain insights into the latest research and findings.

83. Understanding treatments for heterotopic ossification

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Heterotopic ossification, Bone formation, Soft tissue, Bone biology, Drug delivery

Animal types	Life stages
Rats	Adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to use our established model of heterotopic ossification (HO) to understand and explore therapeutic options to prevent and treat heterotopic ossification.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Heterotopic ossification (HO) is a condition in which bone forms pathologically within soft tissues, causing pain, limiting movement and, in the case of amputees, preventing the proper use of protheses. HO may occur following high energy injuries, such as blast,

routine surgeries including hip arthroplasty, and injuries to the brain or central nervous system, which have been shown to cause HO in the elbow joints - the cause of this is unknown. Current preventatives (usually non-steroidal anti-inflammatory drugs or radiotherapy) are often ineffective and come with a range of side effects, and once formed the only treatment for HO is surgical excision of the bone, with recurrence common. There is therefore an urgent need for new, more effective therapies to prevent and treat HO. We wish to further understand and test potential new therapies to prevent or reduce the formation of HO with the potential to significantly enhance patient quality of life.

What outputs do you think you will see at the end of this project?

The primary output of this project will be to identify the most promising therapeutics to prevent and treat heterotopic ossification. These therapeutics operate through different modes of action, meaning that it will also help us to develop future strategies for treatment.

We expect to disseminate our findings through peer-reviewed publications, to compile our knowledge and share it with other researchers in the area. Promising therapies may lead to a new product for clinical use.

Who or what will benefit from these outputs, and how?

In the short term, beneficiaries of the work will be researchers investigating preventions and treatments for HO.

In the longer term, by identifying effective therapies, this project has the potential to identify significantly more effective treatment modalities for HO. Predictors of patients at risk of HO are becoming increasingly accurate, and therefore coupling this with effective prophylaxis could radically reduce incidence of HO. As there are no medical treatments to reverse HO, this project may also provide an alternative to surgical excision.

In addition to improving the physical, psychological and social health of individual HO patients, this project may also impact healthcare systems, such as the NHS, by reducing the need for costly and time-consuming surgeries, physiotherapy and additional care.

How will you look to maximise the outputs of this work?

We will maximise the outputs of this project by collaborating with experts in the field, both in order to study the most promising therapies, but also to get the most information and understanding from our findings. We intend to rapidly disseminate the results of this project, both through presentations and publications, whether positive or negative. We plan to take all appropriate steps to maximise the potential of any effective therapies we identify to make it to clinical use, including protecting intellectual property in a timely manner, and collaborating with partners to realise the potential of our findings.

Species and numbers of animals expected to be used

- Rats: 200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult rats are being used because, unlike mice, following tenotomy they deposit bone with the structural features of human bone. A tenotomy is a small surgical procedure in which the Achilles tendon (heel cord) is cut. Achilles tenotomy induces heterotopic ossification (HO), which is the model we are using to assess new treatments. The bone anatomy of rats is more similar to humans than that of mice (e.g. mice do not have the same nerve and capillary structures within bone, rats and humans do) and pathogenesis of HO in mice is not the same as in rats and humans. We are using adult rats as HO most commonly occurs in adult humans.

Typically, what will be done to an animal used in your project?

Achilles tenotomy will be performed in order to induce local heterotopic ossification. The tenotomy induces inflammation and stimulation of pathological bone formation in soft tissue where bone normally does not present.

All animals will undergo tenotomy surgery. This involves cutting the achilles tendon in one hind leg on one occasion only, whilst the animal is under general anaesthesia. The animal will then be allowed to recover and then will be maintained for 10 weeks while HO develops in that leg. Animals will be administered with therapeutic substances (or control), via injection into the local site, up to a maximum of once per week during this time. Animals will then be humanely killed for postmortem analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Unilateral tenotomy is not expected to cause more than mild alteration in gait; previous studies suggest only mild, short term discomfort associated with the surgery and no long term effects beyond the altered gait. Post operative pain is expected but will be mitigated by appropriate use of analgesia.
Application of therapeutic substances (or control) via injection, is likely to cause minor, transient discomfort.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All animals will experience moderate severity.

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Whilst some elements of HO can be modelled in vitro, the complexity of multiple cell types and organs interacting and differentiating over time to form three dimensional bone within soft tissue cannot currently be modelled in either cell culture or in silico. The use of live animals is thus unavoidable, and essential for the demonstration of therapeutic efficacy in a situation relevant to the human condition. Only mammals have a sufficiently developed immune and musculoskeletal systems to readily compare to humans, and rats are the best model to achieve the scientific aims of this project.

Which non-animal alternatives did you consider for use in this project?

There are currently no alternatives to animal work for the HO model.

Current cell culture models don't have the complexity of clinically relevant HO formation. However, we routinely use cell culture models to identify the most promising therapeutic agents, minimising the number of animals that need to be used and maximising the chances of success in-vivo. In addition to in vitro models, ex-vivo efficacy has been tested (using donated human tissues). Similar non-animal studies will be performed to ensure only the most promising therapeutics are investigated in vivo.

Why were they not suitable?

The primary reason that animal modelling is necessary is that the complex biological interactions between multiple systems and cell types, in three dimensions, changing constantly over time to produce bone within soft tissue cannot be modelled accurately in either in vitro or in silico systems. Part of the problem is that the pathways that cause HO are multifarious and poorly understood. A cell culture system would need to include all of the cells present in the musculoskeletal and immune systems, in the correct spatial location in 3 dimensions, exposed to the correct milieu of stimuli that would create a clinically relevant HO response. We are aware that models are being developed, including three dimensional and co-culture models, but these currently are not sufficient to model the complexity of HO to be able to determine any therapeutic efficacy of our compound.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Numbers of animals have been estimated based on calculations using previous and published data to inform the number of animals required to achieve statistically significant

data in this project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will write a protocol for each experiment, including a statement of the objectives, a description of the experiment, covering the experimental treatments, the size of the experiment (group numbers and sizes), and experimental material, outline the methods of analysis, and identifying opportunities for randomisation and masking of groups.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use as many markers as possible, in order to derive as much data as we can from each animal. We will harvest the maximum amount of tissue and biofluids post-mortem at the end of each experiment, archive anything we cannot immediately analyse, and make unused tissues available to other researchers.
Experiments will be planned so they can be published in accordance with the ARRIVE 2.0 guidelines.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

There are 4 common small animal models of HO.

1. Genetic manipulation, which is complex, requiring several manipulation and breeding steps that requires a significant number of animals to achieve the desired genetic manipulations for study, and these models often lead to a clinically irrelevant phenotype or early mortality. Viable genetic models often require an additional cardiotoxin injection.
2. Models where materials are injected or implanted and bone forms around them are common, however the implanted material often requires artificially high growth factor concentrations, and results in bone formation distant to the area of application. This method does not replicate the clinical formation of HO.
3. Blast amputation models, while the most closely matched to human HO caused by high energy injuries, are high severity and are often associated with high mortality.
4. Achilles tenotomy reliably and consistently produces HO in a similar manner to that seen in humans, minimising the number of animals that need to be used. Animals display minimal adverse effects following the procedure, and in our experience the gait only alters for a short time after surgery.

Of the four models described above, we have therefore decided to use the achilles tenotomy approach as it provides the best physiological approximation of HO while causing the least pain, suffering, distress and lasting harm to the animals.

Why can't you use animals that are less sentient?

We cannot use less sentient species, such as zebrafish or drosophila, as their musculoskeletal systems are completely different to mammals, and so would not represent the human condition. Terminally anaesthetised and embryonic animals are inappropriate as the model requires at least 10 weeks for HO development.

Additionally, whilst a known limitation is that the bone micro-architecture in rodents is not identical to humans, rats are the closest comparison as unlike mice, they have a rudimentary version of the structures seen in the outermost regions of human bone. In addition, most of the preliminary work has been performed in the rats and so use of the same species means previous data can be used to inform direction, rather than needing to repeat experiments in a different species. Our protocols have been established and refined by us and others for over 5 years and is currently the most refined HO model.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have selected the least severe model of HO. Animals will be administered pre-, peri- and/or post- operative analgesia as appropriate under the advice of the Named Veterinary Surgeon. Animals will be closely monitored following surgery and injections of treatments to ensure they have recovered appropriately.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Prior to all experiments, we will consult the PREPARE guidelines checklist, to ensure that valuable data will be generated from the experiment.

The resulting data will be published in open access journals where possible, and in accordance with the ARRIVE 2.0 guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed of advances in the 3Rs by reading the NC3Rs newsletter, attending seminars, webinars and conferences, as well as discussions with the named veterinary surgeon and named animal care and welfare officers. We will also review each experiment on completion, in order to determine any refinements that can be applied in the future.

We will also continuously review the scientific literature in order to identify newly emerging technologies and models that could potentially be adopted to replace in vivo animal use.

84. Antibody production

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Antibodies, Immunisation

Animal types	Life stages
Cattle	Adult
Sheep	Adult
Goats	Adult
Llama	Adult
Camel	Adult
Chicken	Adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To raise polyclonal and monoclonal antibodies in camelids, cattle, sheep, goats and chicken that will provide benefits detailed below. Antibodies produced will be available to academic collaborators and to industrial clients to further their work.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Production of entirely new antibodies to scientific targets of interest that can be used as a) tools to advance basic science, b) contribute towards new drug discovery and development, c) development of clinical diagnostic tools and d) provision of tools for industrial clients to benefit UK economy.

What outputs do you think you will see at the end of this project?

This project will lead to the production and use of polyclonal and monoclonal antibodies in basic science as assay tools, in clinical diagnostic tests and structural biology, and as polyclonal or monoclonal antibodies. Much of our work focuses on specialised antibodies from camelids and cattle and such antibodies has further potential in the development of treatments for life threatening diseases such as cancers and auto-immune diseases.

Another major output is outreach, not only within academia and industry but also public-facing with contribution to events aiming to popularise science the general public and school-aged children and to communicate science to the national and international media.

Who or what will benefit from these outputs, and how?

Our technical service is the only one in the UK that offers this specific antibody technology. UK researchers and our external partners, who rely on us to develop antibodies, benefit the most from our service. While many commonly used antibodies can be bought from commercial suppliers, there's still a need to create new, specialized antibodies for early-stage research and drug development, especially for niche projects.

In 2019, the first therapeutic 'nanobody drug' (called caplacizumab), was approved, and recent technology advances have even led to the creation of 'hybrid' drug molecules, these are likely to be an important part of therapy in the future. So far, the Federal Drug Authority, who regulate all new drugs in the USA, has approved nearly 200 monoclonal antibodies as drugs, and these have become some of the best-selling therapies, showing the huge potential for antibodies in health and scientific progress.

Our work allows UK researchers and partners to develop these antibodies for early-stage research, benefiting both the researchers and their projects. This strengthens research collaborations and supports our long-term goal of being a productive and valuable UK government-approved facility. Additionally, members of our team, including early-career researchers, have won awards and secured funding, which has helped advance their careers.

How will you look to maximise the outputs of this work?

We will maximise the outputs described above by further development of our Antibody Production service. We have recently received initial UKRI Innovate funding to promote the Antibody Production service and investigate expansion of Antibody Production service to further UK academics and partners to develop the technical service for production of bespoke high-affinity antibodies.

Species and numbers of animals expected to be used

- Cattle: 100

- Sheep: 75
- Goats: 75
- Camelids:
  - Llama: 50
  - Camel: 10
- Other birds:
- Chicken: 100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use adult cattle, chicken, sheep, goats and llamas and have previous permission to use camels. All animals will be used at the adult life stage as requisite immune function is optimal and procedures are best tolerated.

Typically, what will be done to an animal used in your project?

Each procedure will involve the following steps:

1. An optional starting control blood sample: At the start, a small blood sample may be taken to measure the baseline levels of antibodies and check blood cell counts. This is done using a small blood vessel near the surface of the skin.
2. Primary Immunisation: The first step in creating antibodies involves injecting a substance (called an antigen) under the skin or into the muscles of animals like chickens, camels, cattle, goats, or sheep. For chickens, the injection is very small and can be given in up to four sites. For the other animals, the injection is larger and can also be done on shaved skin using special devices like a "gene gun" or needle-free injection systems.
3. Booster Immunisation: After the initial injection, more injections (boosters) are given every four weeks to strengthen the antibody response. This can continue until the desired amount of antibodies is produced, but no more than 10 booster shots will be given. In some cases, injections might be more frequent, but the total amount of substance injected stays the same.
4. Test bleeds: Around 10 to 28 days after the injections, small blood samples are taken again to check the level of antibodies. The amount of blood taken will not be more than 15% of the animal's total blood volume in any 28-day period.
5. Final bleed: This step is similar to the test bleed, and happens around 10 to 28 days after the immunisation, following the same guidelines for the amount of blood collected.
6. Antibody harvesting: Once the animals produce enough antibodies, these are collected from their blood, for some animals antibodies can be collected from other tissue such as bone marrow, spleen or lymph node.

What are the expected impacts and/or adverse effects for the animals during your project?

With reference to the steps outlined above:

Steps 2 & 3: Injections and Monitoring. There is a small chance (less than 1%) that animals could develop skin lumps (granulomas) or abscesses after receiving injections. These will be closely monitored by trained staff. If any abscess appears, it will be drained and treated. For certain injections, the skin will be shaved, and any signs of bruising or scarring will be closely watched. A veterinarian will be consulted if there are any concerns.

There is also a very small risk (less than 0.5%) that the injected substance might cause a serious reaction, like anaphylaxis. To minimize this, animals will be injected one at a time, with a 4-hour gap between each, and will be monitored closely for any negative reactions like unusual behavior, loss of appetite, or discomfort. If any very severe symptoms develop, the animal will be killed humanely. If the symptoms are mild or improve, a veterinarian will be consulted for treatment.

Step 4 & 5 & 6: Blood Sampling and Anaemia Risk. There is a small chance (less than 1%) that taking blood samples from larger animals could cause anaemia (low red blood cell count). The procedure is designed to prevent this by limiting how much blood is taken. If signs of anaemia appear, like pale gums or fast breathing, a blood test will be done to check the animal's condition. If an animal develops anaemia, it won't be used again in these studies. If more than 30% of an animal's blood is lost quickly, it could go into shock or even die, but this is very unlikely as only 10% of blood is taken at a time. Some animals could develop chronic anaemia from conditions like parasites, so they will be pre-screened before the study with a small blood test. Animals with low red blood cell counts won't be used.

General: Stress Monitoring: Animals will be trained to get used to the procedures, but some may still experience stress. Stress will be measured on a scale from 1 to 3, with 1 meaning no or minor stress and 3 meaning severe stress. Animals that show mild stress (score 2) will receive more training, and those with severe stress (score 3) may be sedated or returned to their enclosure for further training before being used again.
Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The great majority of animals will only suffer mild severity but instances of adverse reactions to the immunogens may occasionally be seen, resulting in moderate severity. The harvesting of bone marrow, spleen or lymph node will only be carried out in post-mortem and only in cattle.

What will happen to animals used in this project?

Kept alive at a licensed establishment for non-regulated purposes or possible reuse
Rehomed
Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

At the present time, reliable generation of antibodies with a high specificity for the target antigen continues to rely upon the use of an intact, host immune system in vivo, thereby necessitating the use of animals for this purpose. As this project will be produce antibodies for end users, clear communication between requestors, the licence holder, NACWO and technical staff undertaking the procedures is essential. This is necessary to ensure that the need to raise the antibody is justified, that the most appropriate species and methods are used. We use a pre-screening questionnaire as part of our Antibody Production Form; this illustrates the approach taken to establish that use of animals to generate antibodies is required and fully justified i.e. that no alternative source of existing antibody or means of generation are available.

Which non-animal alternatives did you consider for use in this project?

Our Antibody Production service relies on animal use to produce specialised antibodies (such as llama nanobodies and unique bovine antibodies). whilst synthetic libraries can be produced, these rely on initial work in animals. The is also evidence from our work under the previous Home Office Project Licences that 'natural' llama nanobodies produced from an immunised llama library using our technical service have higher binding affinity that synthetic nanobody counterparts.

Why were they not suitable?

Whilst some progress has been made in recent years to develop molecular biology-based approaches to antibody production that do not rely upon the use of animals, these approaches have seen relatively limited success i.e. antibodies to some antigens cannot be raised in vitro, those raised typically lack specificity and/or high-affinity binding.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals proposed to be used is based upon prior institutional experience associated with an antibody service production licence and previous Home Office Project Licences.

A given animal’s immune system will either produce a desired antibody or not. Whether an antibody is produced by a given animal or not is very much dependent upon per animal variability in immune system function and cannot be defined or predicted prior to antigen challenge. Typically, for larger animals, a single animal is sufficient to generate a given antibody response since experience shows that one animal will produce suitable antibodies to the immunogen.

Where scientific purpose permits some choice between suitable species, use of a single larger animal (e.g. camelid) will be preferred. The minimum number of animals will also be used based upon a) the smallest number required to ensure successful production of antibody (not every individual animal will successfully produce antibodies) and b) the amount of antibody required.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have shown that immunization of larger animals such as llamas with multiple antigens can produce sufficiently high titres of antibodies. There is also some evidence to suggest that an animal that has previously provided a good response to antibody generation has a greater chance of providing good responses to future immunogen challenges. Thus, in species where re-use is permitted under this licence, animals that have previously shown a good response will be preferred and so reduce the overall number of animals challenged.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The majority of work under previous Home Office Project Licences is with llama and cattle. Since 2013, we have enlarged our llama herd from 6 animals to a current herd of 20 llamas. This has been achieved in part by establishing breeding within the herd. Each llama can be used for a maximum of 2 procedures/year. Cattle are sourced directly are from shared use projects. We have undertaken a number of pilot studies; for example, studies in larger animals to investigate if intra/transdermal route can also be used to deliver exogenous DNA, RNA, or protein using devices such as a biolistic particle delivery ‘gene gun’ or a needle-less injection system such as Dermojet. We have also optimised use of less invasive adjuvants in pilot studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Whilst a number of species are listed on the present application to provide flexibility for target type, in order to minimise animal suffering, the choice of species for a given case will be based upon the lowest order species for which a) readily available commercial secondary (marker/tag) antibodies are available and b) are compatible with the experimental purposes.

Why can’t you use animals that are less sentient?

Provision of specialised antibodies is reliant on use of different animal models. Thus, single domain antibodies, which form a larger part of the antibody repertoire specifically in camelid species can be engineered to produce nanobodies. Similarly, bovine antibodies have unique characteristics which also make them attractive for development of therapeutics.

Camelids, cattle, sheep, goats or chicken will be immunised with a variety of antigens to raise specific antibodies. Antibodies from these species make them highly suitable for use in a variety of immunological detection techniques. The choice of species will be made on the basis of the needs of the requestor, the nature of the immunogen and the need to use the lowest order species possible to achieve the stated aim(s). Considerations include the amount of antiserum required, the proposed application and the availability of complementary reagents (e.g. commercially available secondary conjugates). It must also be considered whether the immunogen is likely to result in the animal producing antibodies that will bind to the endogenous molecule to cause possible adverse effects in which case a more distantly related species would be used. In cases where multiple species are suitable for a given application to achieve the stated aim(s), the lowest order species will be chosen.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

During our previous Home Office Project Licences, for some individual llamas, multiple antigens were used (maximum of 4 injections, each at different site). Initial evidence suggests that this approach produced a good titre of antibody to each antigen. Where appropriate, similar multiple injection will be used to reduce animal use.
Suffering will be minimised by sequential injection of immunogen in successive animals in order to reduce the harm done is any systemic adverse effect was produced. During previous Home Office Project Licences we have refine use of proposed adjuvant further. Thus, we have used GERBU LQ, and now GERBU Fama in llamas and Montanide in cattle as a particularly mild, non-toxic and fully biocompatible adjuvant which is highly concentrated for best effect with small injection volumes. These refinements have reduced the use of more irritant adjuvants such as Freunds Complete Adjuvant or Freunds Incomplete Adjuvant (or other adjuvants liable to induce granulomas). Suffering has been further minimised by careful consideration of injection volumes used, needle gauges used, blood volumes drawn via consultation of NC3Rs guidelines and strict criteria by which suitability for re-use of camelids, cattle, goats and sheep will be assessed continually. Clearly defined endpoints for all procedures also prevent the possibility of undue suffering and minimises any necessary discomfort experienced by the animals. We will continue to seek and exploit similar opportunities in the future.
Once a previously unavailable antibody has been produced, the use of in vitro or synthetic techniques to produce further antibodies will be undertaken where scientifically possible.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will consult published best practice guidance in 3Rs refinement overseen by an Institutional Animal Care and Use Committee (IACUC) and guided by the ARRIVE guidelines for transparent reporting of animal research data. We also consult a reference paper for use of research area-gold standard ARRIVE experimental design and data analysis (Lilley et al. 2020 ARRIVE 2.0 and the British Journal of Pharmacology: Updated

guidance for 2020. Br J Pharmacol. Aug;177:3611-3616. doi: 10.1111/bph.15178). We follow transparent reporting of animal research data according to the ARRIVE checklist in work with animals for antibody production.
Similarly, we adhere to Home Office Animals in Science Committee “Antibody report by the Project Licence Strategic Review subgroup” Oct 2022, in our work with animals for antibody production.
For consideration of injection volumes used, needle gauges used, blood volumes drawn, we have consulted NC3Rs guidelines (https://nc3rs.org.uk/3rs-resource-library/blood-sampling/blood-sampling- general-principles)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Due to the nature of our technical service to academia, research institute and pharmaceutical industry partners, we are highly aware of keeping informed of the latest advances in the 3Rs with regard to optimising use of animals and associated techniques. We are supported by a local User Group who hold regular meetings with a varied agenda, which includes: to reinforce the Culture of Care including user surveys and the holding of regular 3R seminars on the subject of ethics, training and assessment, animal colony health screens etc.
All work is supported by Animal Welfare and Ethical Review Body (AWERB) reviews of all Project Licences, the AWERB Committee consists of members (including NACWO, NVS who can advise on latest advances in the 3Rs and ensure that Home Office Project Licences adhere appropriately to current advice.

85. Identification of drugs and drug targets with the capacity to modify skeletal homeostasis

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Bone, Cartilage, Joints, Homeostasis, Therapeutics

Animal types	Life stages
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult
Rats	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to advance our basic knowledge of skeletal system remodelling, repair and regeneration in order to translate understanding of mechanisms to new therapeutics for disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Failure of the skeletal system in the two most prevalent conditions, namely osteoporosis and osteoarthritis, produces massive socioeconomic and healthcare cost and their incidence is rising due to the ageing population. Paradoxically, inherited/genetic musculoskeletal diseases are also becoming more important in companion animals. With a view to finding new ways to restore function and lifelong health, our work aims to unravel how skeletal tissues achieve, retain and lose function. We will also assess therapeutics with the potential to reverse or halt disease processes.

What outputs do you think you will see at the end of this project?

New therapeutic entities that can modulate disease processes for clinical translation An understanding of disease processes and tissue homeostasis
Publications relating to the identification of drugs and drug targets that control skeletal homeostasis Presentations at national and international conferences
We aim to find approximately 10 targets, trial 5 drugs and predict most would show efficacy

Who or what will benefit from these outputs, and how?

Through this program of work, we expect to identify novel drugs and drug targets that can modulate human and animal skeletal homeostasis and disease. The investigation of rodent models of disease is expected to improve our understanding of the molecular and cellular processes that govern tissue homeostasis. This will give rise to unique disease intervention points that can be targeted with biological and chemical entities. It is also expected that this study will inform us on novel clinical markers (biomarkers) that are representative of disease state, progression and response to treatment. These markers will be translated to clinical studies investigating novel therapeutics in skeletal disease. These results may be used to support clinical trials as well as to add to our understanding of disease pathogenesis. This will have benefit to both human and animal patients. Reagents/models/techniques developed in this project are likely to benefit other researchers working in the field.

How will you look to maximise the outputs of this work?

We will ensure dissemination of our work through collaboration with other groups working in the same and distinct areas of research, and through presentation at national and international meetings. We will endeavour to publish all data from the project. The PPL holder is a trustee on the Rare Bone Disease Foundation and Bone Research Society which will aid dissemination to non-scientists.

Species and numbers of animals expected to be used

- Mice: 5000
- Rats: 300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The rodent (mouse and rat) animal model has been proposed herein to study skeletal homeostasis due to a number of reasons. Firstly, the relatively short lifespan of rodents allows the study of age- related disease over a period of time that is compatible with a research project such as this.

Furthermore, genetic modification along with immunocompromised animals have allowed functionality of specific substances in the generation of the skeleton to be described. The use of rodents for the investigation of skeletal disease has also proved a successful strategy, further de-risking the choice of the proposed models. Indeed, rodents exhibits age-related degenerative skeletal diseases such as osteoporosis and osteoarthritis which are equivalent to that seen in the human population. We will use juvenile and adult rodents to ensure we have the capacity to assess both childhood and adult disease.

Typically, what will be done to an animal used in your project?

Rodents might receive substances (biologic e.g. protein or cells/tissue; chemical e.g. small molecule drug; or a tracer e.g. chemical that attaches to tissue or cells) with or without a surgical or mechanical intervention to modify tissue homeostasis. Some animals may be analysed for gait changes or hearing modification (not regulated procedures).

What are the expected impacts and/or adverse effects for the animals during your project?

The rodent models proposed have no/few clinical signs associated. Furthermore, it is very unlikely that rodents will reach a disease stage where humane endpoints are necessary. Examples of potential adverse effects include transient pain from ovariectomy in protocol 2 (up to 24hrs) and transient mild lameness from loading in protocol 2 (up to 48 hours) and joint surgery in protocol 3 (for approximately 7 days). Adverse effects associated with the administration of substances are likely to only be transient. Foxn1nu rats display an altered immune status, this is necessary to allow xenotransplantation of cells. The Sost-/- mouse may display pathological changes related to sclerosteosis disease, but are still largely unknown. Mice may suffer deafness, however this is currently under investigation. The STR/Ort mouse displays pathological changes related to osteoarthritis, which are observed by imaging (CT and histology). However, these do not result in any welfare concerns and no visual signs are present.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild (30%)
Moderate (70%)

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The processes involved in modifying skeletal architecture and mass and those involved in the degeneration of the joint that can only realistically be replicated in vivo due to the interaction of multiple cell types and the immune system.

Which non-animal alternatives did you consider for use in this project?

In vitro organ culture systems appear capable of at least partly replicating some of the events whereby these mechanical stimuli are applied and may therefore be useful in examining the immediate and short-term responses to such application, currently in only individual cell types. Individual cell types can be exposed to substances in vitro but these are unlikely currently to replicate the effects of such exposure in vivo due to effector cells also being mechanosensitive or in constant communication with neighbouring cells to control their biology. Attempts to bridge this in vitro divide are actively being explored, through organ-on-a-chip type approaches and the 3Rs Crack-it agenda but these are still in their early developmental stages. Monolayer cell culture can sometimes be used to replicate selected aspects of both of these types of responses. These in vitro and cell culture-based alternatives have been, and will be, used by us as replacements wherever possible to examine some selected aspects of the responses we aim to more fully decipher. Examples include the exploration of the behaviour and response of primary osteoblasts and osteoclasts, osteocyte cell lines and organ cultures of embryonic metatarsals (mostly requiring derivation from animal limbs ex vivo). We have fully acknowledged their strengths, reviewed their use for others, but are aware and appreciate their limitations.

The following databases were searched for alternatives:

The John Hopkins Centre for Alternatives to Animal Testing (http://altweb.jhsph.edu) Animal Welfare Information Centre (https://www.nal.usda.gov/programs/awic) European Centre for Validation of Alternative Methods (http://ecvam.jrc.it/index.htm)
Fund for the Replacement of Animals in Medical Experiments, FRAME (http://www.frame.org.uk/) NC3Rs (https://www.nc3rs.org.uk/3rs-resources/search?topic%5B0%5D=504)

Why were they not suitable?

In vitro cell and organ culture systems cannot replicate the discrete interaction of cells and tissues to elicit changes in skeletal architecture and mass, or the integrated response of the joint as an organ towards degeneration. These in vitro approaches also fail to produce the range of structural abnormalities in skeletal tissues that can be seen, sometime after, in response to abnormal homeostasis. Monolayer culture can fall short of providing the

integrated, organ-level, physiologically intact environment in which such responses are normally coordinated.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Numbers of rodents will be used to generate a robust, statistically significant result that is of biological/disease importance. However, we will also ensure that we do not use any more animals than are necessary to demonstrate this effect. These numbers can be determined, by prior understanding of the effect that is being assessed and the variation within the animal population and experimental intervention being examined.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We review previously published research to gain insights into the range of variation within the intended study model and the significance of the effect size we aim to achieve. This effect size must hold biological or disease relevance for us to proceed. The NC3Rs Experimental Design Assistant will provide algorithm-generated feedback on potential adjustments, such as identifying sources of bias or nuisance variables, and visually representing experimental designs for group or external discussion when appropriate. We leverage additional online resources, such as GLIMMPSE, to ensure optimal sample sizes for maximizing the likelihood of a positive outcome, particularly in studies involving repeated measurements, thereby enhancing the statistical power of our comparisons. Whenever feasible, we utilize stored tissues from prior studies to minimise the need for animal use.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will only breed animals for specific experiments or to maintain a colony. In novel experiments, we will conduct pilot studies, where appropriate, to help inform our future experimental design. Wherever possible, surplus tissue from animals will be shared with other researchers.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We have chosen to focus primarily on rodents. This decision has been made as it will provide us with the potential to explore the role of specific drugs and drug targets in the skeletal response along with the use of mutant and transgenic mouse models for further investigation. Mice are well respected as the animal model with the lowest neurophysiological sensitivity for the study of skeletal development that is equivalent to humans, which have been shown to be relevant for the study of drug targets (including RANKL and Sclerostin) for the development of osteoporosis therapeutics.
Animal suffering will be limited in our studies by our strict monitoring of severity limits and our use of protocols that do not produce excessive trauma or suffering. The alternative strategies which others have used to attain similar endpoints frequently involve surgery and our use of surgical approaches will be kept to a minimum. Appropriate pain relief during our protocols will be achieved through use of tailored levels of analgesia. We have also made more routine, the monitoring of changes in animal gait through the use of short-term video recordings for locomotor analysis. These allow us to determine the relationship, for instance, between the emergence of gait asymmetries and the onset and advance of osteoarthritis.

For administration of substances we take great care to consider fully the routes of administration to limit the number of occasions on which an animal is handled and dosed. A prior example of our refinement in dosing strategy is the switching from oral gavage to supplementation of the drinking water for a specific compound. This refinement was made possible by our keeping abreast of the most recent advances in the field and by seeking out collaboration from the manufacturer, the industry partners and others academic researchers using the same compound. We will continue to seek out the most up to date information that will facilitate similar refinement in our approach.

Why can't you use animals that are less sentient?

Rodent models allow us to examine responses in skeletal homeostasis, which are crucial when evaluating treatments and disease mechanisms in humans or other mammals. Mammalian models are needed to accurately assess the effects and efficacy of treatments. We may administer treatments at a juvenile stage, so efficacy can be assessed towards childhood disease.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Rodents will be closely monitored for signs of suffering or welfare problems through regular observational assessment and in-study weighing, however, we do not anticipate specific harms as the models we are studying do not elicit chronic pain symptoms or are associated with welfare issues. Any animal showing signs of an adverse effect will be assessed daily using our study specific score sheets, which has humane endpoints defined. We will not perform procedures that are associated with expected pain, other than transient discomfort or that which can be easily controlled with post-surgical analgesia.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow guidance generated by the NC3Rs in terms of husbandry.
We follow LASA guidelines (wherever appropriate) for administration routes and blood sampling protocols.
Work conducted within the BSU, and supported by local staff, is performed with attention to the Culture of Care, promoted by the PREPARE guidelines, which is fully supported by this research team.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will conduct regular 3Rs assessments for our work and utilise online tools and information and advice from the NC3Rs including their Resource Library and Resource topics. We will communicate with our NC3RS liaison officer and institute changes whenever we can to improve animal welfare, reduce numbers of animals required and refine the methods. Additionally, the licence holder sits on the scientific advisory board of FRAME and as such is constantly kept up to date with advances in the 3Rs.

86. Impact of pharmacological and environmental exposures on the developing epigenome

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Gene regulation, Epigenetics, Development, Advanced therapeutics, Maternal environment

Animal types	Life stages
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to determine how pharmacological (such as drugs taken by the mother) and environmental (such as changes to the mother's diet) exposures experienced during pregnancy impact the short- and long-term health of the resulting offspring.
Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

When a woman is pregnant, she can experience changes to her environment, for example her diet or the chemicals she comes into contact with, or be required to take a drug, and all of these can impact her baby's development. Some of these exposures have been linked to negative outcomes for the baby, including pregnancy loss, low birth weight and disease in the child's later life. Within the UK, it is estimated that 250,000 pregnancies a year end through miscarriage and another 30,000 women are admitted to hospital due to pregnancy losses from other complications. In 2021, around 3,300 women made the difficult decision

to end their pregnancy for medical reasons. Pregnancy complications can be extremely isolating and affect the emotional wellbeing of women and their families. Our work aims to produce new tools to allow us to understand how different exposures impact the health of the developing baby.

DNA (deoxyribonucleic acid) carries all of the information, split into units called genes, required for the cells in our bodies to form and function. The DNA in every cell in our body is the same but each cell is able to turn on (express) or off (silence) different combinations of genes to have a unique identity.

Epigenetics refers to the study of how the activity of different genes is controlled. DNA methylation is a label that is added to DNA to control gene expression. The location of DNA methylation can change over time. The pattern of different epigenetic labels on DNA is referred to as the epigenome. In this project, we will generate new mice with a built-in "reporter", which acts as a glowing tag inside the animal or cell. This will allow us to understand when specific genes are active and when DNA methylation marks are changing.

We will use these mice to understand when DNA methylation changes during pregnancy. By exposing pregnant mice to changes in their environment (e.g. diet) or to different drugs, we will be able to use the built-in reporter to observe when these exposures cause changes to DNA methylation and gene expression. We will also study the impact of these exposures on the health of the developing offspring. We will then observe the health and DNA methylation status of these mice during their early and adult life, once the exposure is removed. This will allow us to understand if the offspring is able to recover or if the exposure has a long-term impact on health. To understand if the offspring can pass on any of the changes in DNA methylation status to future generations and what this means for the health of those generations, we will breed the mice exposed during pregnancy. Ultimately, this work could inform our understanding of which environmental exposures (e.g. diets or chemicals) during pregnancy are harmful for the developing baby and which drugs are safe to be taken during pregnancy.

What outputs do you think you will see at the end of this project?

A successful outcome of this project would be the generation of new mice with built-in reporters to allow us to understand the impact of different exposures experienced during pregnancy on the epigenome and life-long health of the offspring. This has the potential to lead to a new understanding of the safety of different drugs and chemicals during pregnancy and provide new ways to design medications for the treatment of conditions experienced by pregnant women. Our findings will be presented at international conferences and/or published in high-impact scientific journals. In addition, our lab will be engaging with the public through communication and outreach activities, in science fairs and by visiting schools.

Who or what will benefit from these outputs, and how?

The benefits that are likely to be realised by successful completion of the work detailed in this application include the production of new mice with a sensitive reporter to detect long-term changes to the epigenome during development and adult life. This will allow a greater scientific understanding of the impact of the mother's environment on the developing

offspring and the ability for the epigenome to recover after the influence of pharmacological or environmental exposures.
This system could also be used to screen the effects of different exposures commonly experienced during pregnancy to determine the true long-term impact on the offspring. In the long-term, this could have a huge impact on our understanding of which drugs/compounds are safe for pregnant women to consume and which have unintended negative effects on the offspring, particularly those that only materialise in later life.

How will you look to maximise the outputs of this work?

The output of this work will be maximised by active collaboration with leading experts in the study of the epigenome. Their advice will be sought during the entire project and scientists from multiple labs will contribute to the analysis of the data produced during this project to ensure the maximum information can be obtained. Any new knowledge gained during the course of the project will be shared with other researchers, through the publication of research papers and sharing information at conferences and scientific meetings. This will include sharing information about any unsuccessful approaches or refinements that can be made to approaches to ensure the output of similar studies is maximised in the future.

Species and numbers of animals expected to be used

- Mice: 15000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The mouse will be the main experimental model used to investigate the impact of pharmacological and environmental exposures on the epigenome of the developing offspring. In vitro models (methods to study the behaviour of mammalian cells in a controlled environment in a non-biological context) cannot be used to study the biological relationship between the mother and the developing offspring. They are, therefore, not suitable to fully answer questions related to the impact of exposures during pregnancy on the developing offspring, such as those detailed in this application.

We will create new mice that harbour reporters in genes in the mouse genome that will enable us to detect changes to the epigenome following different exposures during pregnancy and to uniquely determine if these changes persist into adulthood. Our goal is to test different exposures experienced by the mother during pregnancy, such as advanced therapeutics (drugs used to treat a variety of different disease/conditions and that are predicted to impact the epigenome), changes to maternal hormone levels and changes to maternal diet, to determine the effect on the epigenome of the developing offspring and to determine if the epigenome is able to recover following such exposures. Our new reporter mice will be studied during pregnancy and from embryonic stages through to adulthood, up to the age of 15 months.

Typically, what will be done to an animal used in your project?

This PPL aims to study the impact of pharmacological and environmental exposures during pregnancy on the developing epigenome. In order to do this, we will need to make, breed and maintain genetically altered mice, in which reporters are introduced into genes controlled by DNA methylation. These mice are not predicted to have any negative health effects but the animals will be monitored by trained technicians to ensure this is indeed not the case.

Mice, including dams (pregnant female mice), will be exposed to advanced therapeutics. In this PPL, advanced therapeutics refers to drugs that are used to treat a variety of different human diseases/conditions and that are predicted to have effects on the epigenome. Dosing strategies and administration routes will be determined in accordance with published literature or through the use of small pilot studies to determine the correct dose to minimise the impact on the dam. Examples of administration routes that could be used to supply the therapeutics are intraperitoneal injection (IP - injection into the body cavity), intravenous injection (IV - injection into the blood stream) or subcutaneous injection (SC - injection under the skin).
For diet studies, dams will be exposed to different diets or a calorie-matched control diet. These mice may be exposed to additional dietary supplements, such as folate. For studies into the impact of altering maternal hormone levels, dams will be exposed to substances known to change hormone levels, using administration routes recommended by published literature. Typically this will involve oral administration, either in food, drinking water, through oral gavage or dissolved in an edible substance, such as a palatable jelly or syrup. To determine hormone levels, blood samples may be taken from the dam. Blood sampling will be conducted according to specific criteria, which includes not collecting more than 15% of the total blood volume.

Dams and their offspring may be used for non-invasive imaging, such as IVIS Spectrum imaging. IVIS Spectrum imaging is a non-invasive imaging system that allows fluorescent proteins to be detected inside live animals. Fluorescent proteins are glowing tags that are used in reporter mice to observe changes in gene expression. In this PPL, the fluorescent proteins are used to determine if genes controlled by DNA methylation are expressed following an environmental or pharmacological exposure. During imaging, mice will be kept under anaesthesia to prevent distress or the need to repeat the procedure due to movement.

To understand the impact of pharmacological and environmental exposures during pregnancy, some dams will be killed humanely at specific time points. This will allow the developing embryos to be collected from the dam and analysis of gene expression levels, DNA methylation and protein levels to be conducted using their tissues. Multiple tissues will be collected from the dam and each embryo and any spare tissues will be correctly stored and made available to other researchers. This will provide detailed information about the impact on the epigenome of the developing offspring at the time of the exposure.

To assess the impact of pharmacological and environmental exposures during pregnancy on the relationship of the mother and offspring, some dams will undergo behavioural tests after birth. This may involve separation of the dam from her pups for defined periods of time, before observing key maternal behaviours on return of the dam to pups. An example of a maternal behaviour to be observed is the time taken to retrieve all pups and return them to the nest. In some cases, behavioural tests will require one brief exposure to a

stressor, such as reduced bedding, typically for no more than 10 minutes. All assessments of maternal behaviour are not expected to cause any long-lasting negative effects on either the dam or pups. In some cases, pups may be separated from the original dam and fostered to a different female. This would occur following rare cases of complications during pregnancy, resulting in the requirement for removal of the pups by c-section and humane culling of the dam, or in cases when the long-term health and behaviour of the pups needs to be assessed independently of any impact of the exposures on maternal behaviour. Whenever possible, however, the pups will be kept with the original dam.

To assess the impact on metabolic processes (chemical changes that take place in an organism to produce energy and basic materials needed for important life processes), animals may receive substances, such as glucose or insulin, which will typically be administered once into the abdomen, followed by blood sampling of one drop, using a blood sugar monitor similar to that used by diabetics. Up to 6 blood samples will be taken from the tail vein over 4 hours.

To understand the impact on behaviour and brain function, some animals will undergo 1, 2 or sometimes 3 behavioural tests designed to assess motor skills, anxiety levels and spatial navigation.

What are the expected impacts and/or adverse effects for the animals during your project?

The vast majority of animals will undergo well-established procedures and will experience no adverse, long-lasting effects.

During the project, mice can undergo embryo transfer or IVF (in vitro fertilisation, in which an egg is fertilised by sperm outside of the body, prior to transfer of the fertilised egg into the body of a mouse). Mice that will undergo surgical embryo transfer will experience transient post-operative pain and discomfort. Animals that go through IVF will experience mild transient discomfort and no lasting harm. In addition, mice might be undergoing vasectomy surgery and will experience short-lived post- operative pain and discomfort.

During this project, we will be creating new genetically altered mice, in which reporters will be introduced into genes controlled by DNA methylation. Whilst these mice are not predicted to show any adverse effects due to the genetic alteration, the mice will be closely monitored by trained technicians. We study genes that have the potential to effect growth and development. New genetically altered mice will be monitored for slow weight gain in early life and impacts on body condition.

Animals will experience pharmacological exposures. Injections may cause short-term stress to the animal or may cause reactions at the site of the injection. Mice will be trained to become used to handling multiple times a day in advance to reduce the risk of stress from the handling required to perform an injection. To reduce the risk of a reaction at the site of injection, we will inject at a different location on each occasion, when multiple injections are required. When the pharmacological exposure occurs during pregnancy, in rare cases, it may cause pregnancy loss or distress during birth. All pregnant mice will be monitored daily and appropriate measures to reduce stress, such as providing a soft diet, extra nesting materials or warming pads, will be implemented.

Before starting with a full study, we will use pilot studies to assess any potential adverse effects on the new mutant mice compared to the wild-type. During these pilot studies, we will be assessing the dose range and frequencies (number of injections, days of administration, concentration of the different agents) regime for the different pharmacological exposures.

To fully characterise the impacts of different pharmacological and environmental exposures on life-long health, animals may experience blood sample collection from superficial vessels (saphenous veins) and this will cause mild and transient discomfort. Animals may undergo imaging techniques, requiring the animal to be under anaesthesia. Mice will have no more than ten anaesthetics throughout their lifetime and only one in a 24-hour period. Mice that do not fully recover from the anaesthesia within 24 hours (eating, drinking and returning to normal behaviour) will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Sub-threshold - up to 60%.
Mild - up to 25%.
Moderate - up to 15%.

What will happen to animals used in this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The experiments are performed to assess the health of whole animals. The observations will include multiple organs and cell types within the living animal. These observations cannot be made using cells in a dish, as these are simplified systems and allow the study of very few, isolated cell types. It has also been shown that the epigenome of the cells in these systems differs greatly from their natural state within the living animal and therefore cannot be used to answer the scientific questions detailed in this application.
A large focus of the experiments will be on mechanisms and pathways in a developing animal, including both embryonic and adult stages. It is important that we are able to observe different time points throughout development. We will also study how the mother and their offspring interact during pregnancy, which cannot be modelled using a cells in a dish system.

Which non-animal alternatives did you consider for use in this project?

We use non-animal alternatives where appropriate before moving on to use animal models. Such alternatives include cell lines, which we can use to mimic as much as possible developmental processes. These will allow simpler questions to be answered. Initial experiments to test some of our hypotheses and choose the best pathways to focus on will be conducted in these cell lines. This will allow us to reduce the total number of animals we use.

We will also use publicly available resources and re-analyse existing data, where possible.

Why were they not suitable?

Very often, non-animal alternatives are not complex enough and do not show the interactions occurring in a physiological (normal) context. This prevents scientific questions regarding the impact of exposures experienced during development to be fully answered. The epigenome in the cells within these systems also differs greatly to those within a living animal and so to be able to conduct experiments that produce relevant data we must use animal models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of animals has been estimated based on our own work and that of our collaborators, who have been working in this research field for the past 30 years. We estimated the numbers required for the project from these past experiments, which allowed us to determine our sample sizes.
The research requires the use of a large number of animals. This is, in part, due to the nature of the work, which focuses on key genes controlled by DNA methylation. These genes are known to show differences between the copies inherited from the mother or from the father. Due to this, we need to follow the transmission from the mother and from the father in separate breeding pairs in order to see these key differences, which doubles the animal numbers required for some experiments.
In addition, it is important to study both male and female animals. It is known that environmental and pharmacological exposures impact the two sexes in different ways and to different extents and, therefore, it will be important to include data from both male and female mice in our analysis.

We have designed new mice with a built-in reporter, which will allow us to determine the long-term impact of exposures during pregnancy in a manner that has not been possible before. The nature of the reporter, however, means that many experiments only focus on a specific combination of genetic changes, which is not carried by all animals that are born. For these cases, when animals that are born will not be used in experiments, we have a sophisticated communication platform within our lab and our facility to ensure maximal usage by other researches. For example, for breeding, pilot experiments and tissue

collection. We will also use the NC3R's breeding and colony management resource to ensure we are following best practice.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We used online tools, such as the PREPARE guidelines (https://norecopa.no/PREPARE), the NC3Rs website (https://nc3rs.org.uk) and the NC3Rs EDA (https://www.nc3rs.org.uk/experimental-design- assistant-eda). We also consulted experienced biostatisticians during the initial experimental design phase to determine the correct sample sizes. In addition, we consulted our collaborators, who are experts in this research field, when designing new experiments to gain their additional expertise and advice.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use:

1. Efficient breeding techniques - We will work closely with the animal technicians in our facility, who will advise us if breeding pairs are not performing optimally. All breeding information will be recorded, in our online colony management system, allowing us to notice immediately if breeding pairs are experiencing any problems.
2. Sharing animals and tissue samples - We will communicate with other researchers in our facility to share animals and tissue samples. All animals are recorded in a colony management system, allowing animal users to easily check when animals are available, and we will also communicate this information via a shared email. We will record and correctly store all tissue samples and share this information with our collaborators and other researchers. This means that others do not need to generate additional samples, reducing animal numbers across projects.
3. Preservation of mouse strains - We will freeze sperm and preimplantation embryos from all mouse strains. This will allow us to reduce animal numbers from mouse strains we are not planning to use for at least a year as we will not have to maintain active breeding colonies.
4. Pilot studies - We will use pilot studies, with a small number of animals, to determine whether our experiments are then required in larger numbers to answer our scientific questions.
5. Advanced technologies to generate new mouse lines - Our new mouse lines will be generated using CRISPR technologies, which can generate mutations (changes to the DNA sequence) using a single targeted event. The development of these technologies have resulted in the requirement for far fewer mice, have increased the rate at which new mouse models can be generated and reduced the requirement for extensive breeding schemes.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Throughout this project, we will use mice as an experimental model since the mouse genome has been well-characterised. This allows us to genetically manipulate the genome and thus generate new sensitive mice with a built-in reporter, which will allow us to study the impacts of pharmacological and environmental exposures experienced during pregnancy on the DNA methylation status and health of the developing offspring. The genetic changes we propose in this project will allow us to introduce fluorescent proteins that act as a label for the expression of key genes controlled by DNA methylation. These changes are not predicted to have any negative impact on the health of the animals.
In order to study the impact of pharmacological and environmental exposures during pregnancy on the developing offspring, we propose to expose pregnant dams to advanced therapeutics, altered diets or dietary supplements designed to alter maternal hormone levels. The dosing strategies and administration routes will be chosen to minimise any pain, suffering or distress experienced by the dam. We do not predict that the animals will experience any long-lasting harm. We will implement monitoring, assessment and care techniques that have been perfected by our collaborators over years of experience.

Why can't you use animals that are less sentient?

Our research focuses on mammalian gene function, making the mouse the best model for our work. Our work aims to investigate the impact of pharmacological and environmental exposures on the developing epigenome, with a particular focus on DNA methylation and the process of genomic imprinting. Genomic imprinting is the process by which only one copy of a gene in an individual (either from their mother or their father) is expressed, while the other copy is suppressed. The epigenetic process of genomic imprinting does not exist in less sentient species, such as zebrafish, and therefore we cannot use these alternative species for our research. As we aim to investigate the impact of exposures throughout the entirety of development and post-natal life, we cannot study immature life stages alone.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice will typically be group housed, with enrichments such as tunnels and nesting materials, to minimise welfare costs for the animals. We will discuss new and improved enrichment and housing options with our NACWO to ensure the best possible care for the animals. We are also part of an internal colony management resource group.
When handling pregnant animals, additional care will be taken. We will use cupping and/or tube handling to ensure that the weight of the pregnant animal is properly supported. All mice will be regularly handled prior to any protocol or the induction of pregnancy to ensure that they are used to handling, thus reducing any possible stress to the animal. Following any surgery, or if deemed of benefit to the health and wellbeing of the animal, soft bedding will be supplied and pain relief will be administered. Advice will be sought from the NVS. Established protocols will be used and we will discuss these protocols with experienced collaborators to minimise welfare costs for the animals. We will carry out pilot studies before starting procedures with larger numbers of animals. Any animal that has been through a protocol will be monitored at regular intervals. For example, we will use score sheets for procedural changes, weigh the animals and provide heat mats when this would

benefit animal recovery. When appropriate, pain management such as the use of palatable substances and training for voluntary treatment will be given to the mice.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We use the ARRIVE and PREPARE guidelines to ensure our experiments are performed in the most refined way. These guidelines provide advice regarding study design, sample sizing, randomisation methods, blinding, outcome measurements and statistical methods. Whenever possible, we will incorporate the advice in these guidelines into our experiments.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We subscribe to the NC3Rs e-newsletter to remain informed about advances in the 3Rs and any workshops we can attend to increase our knowledge. We will also regularly discuss how to implement new approaches and best practices in the 3Rs with the Named Persons at our institution.

87. Cell biological processes linking metabolic disorders and neurodegenerative disease.

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

energy homeostasis, obesity, zebrafish, neurodegeneration, cognition

Animal types	Life stages
Zebra fish (Danio rerio)	Embryo and egg, Neonate, Juvenile, Adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim is to understand mechanisms that lead to metabolic disease and their co-morbidities with special emphasis on the association between obesity and neurodegeneration/ dementia.
Disruption of molecules or pathways that control appetite and/or body weight will be studied in zebrafish.

A secondary aim is to identify small molecules that regulate endocrine and immune responses in the brain to reduce obesity-related neurodegeneration.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Obesity results from an imbalance in energy homeostasis such that the energy consumed is higher than the energy utilized. This contributes to an excessive fat accumulation in the body. Obesity is associated with multiple metabolic and endocrine disorders including hyperlipidemias, metabolic syndrome, diabetes, and cardiovascular diseases. Further, obesity represents a risk factor for the development of neurodegenerative diseases such as dementia at older ages. With the World Obesity Federation estimating there will be 206 million children and adolescents aged 5-19 years living with obesity in 2025 and 254 million in 2030, and that 51% of the world, or more than 4 million people, will be obese or overweight within the next 12 years, obesity represents a major risk for public health across the life course. Although, diet is a major contributor to obesity, obesity has a multifactorial etiology, with strong evidence for genetic factors contributing to vulnerability to this disease. This project aims to gain insight into the cell biological pathways contributing to vulnerability to obesity, the link between obesity and neurodegenerative disease and possible gene x diet interactions as well as possible therapeutic and/ or preventative interventions.

What outputs do you think you will see at the end of this project?

Expected outputs include:

1) Generation of animal models to study genetic variants associated with human metabolic disease.

2) Establishment of cell biological processes underlying the association between metabolic disease, neurodegeneration and cognitive decline.

3) Greater understanding of the interaction between genetic and dietary factors contributing to obesity, neuroinflammation and neurodegenerative disease.

4) Identification of small molecules and/or dietary factors that regulate endocrine and immune responses in the brain to reduce obesity-related neurodegeneration.

5) 5 or more publications.

Who or what will benefit from these outputs, and how?

This project will have short term benefits for the academic research community and longer term benefits for those suffering disorders resulting from metabolic disease and obesity. Ultimately, the predicted long term benefit is reduced morbidity and the prevention of human neurodegeneration and death.

<u>The academic community will have short term benefits from:</u>

Output 1-3. Generation of animal models to study genetic variants associated with human metabolic disease, Establishment of cell biological processes underlying the association between metabolic disease, neurodegeneration and cognitive decline. Greater understanding of the interaction between genetic and dietary factors contributing to obesity, neuroinflammation and neurodegenerative disease.

How :

o Model Validation: Establishes zebrafish as a valid and effective model for studying the developmental and physiological roles of genetic variants associated with obesity and metabolic disease. Establishes specific GAA lines for this purpose.

o Increased Knowledge Base: Immediate enhancement of scientific understanding regarding the function of specific genetic variants in developmental and physiological processes in relation to metabolic disease in a model organism closely related to human biology.

o Increased research efficiency: The success of this project will illustrate the increased efficiency, and refinement in animal use, provided by the use of larval zebrafish for the study of metabolic disease.
Objective 4. Identification of small molecules and/or dietary factors that regulate metabolic disease, endocrine and immune responses in the brain to reduce obesity and obesity-related neurodegeneration neurodegeneration.

o Identification of small molecules that prevent phenotypic changes resulting from GAA associated with metabolic disease will be of benefit to academics and clinicians as well as patients as it will give information regarding the cellular processes underlying metabolic disease-related morbidity and will identify candidate molecules that can be used to develop treatments for conditions associated with metabolic disease (including neurodegeneration).

Objective 5. Publication of Research Findings: How:

o Knowledge Dissemination: Immediate dissemination of new knowledge through publications, not only contributing to fundamental knowledge within the global scientific community but also highlighting the utility of zebrafish in modelling human diseases, thereby contributing to the refinement in animal usage which is a priority to society as a whole.
o Clinical Insight: Provides clinicians and researchers with the latest findings, potentially influencing current therapeutic strategies and patient care, emphasizing the relevance of animal models in translational research.

<u>Clinicians and patients and general public will have long term benefits from:</u>

Output 1-3. Generation of animal models to study genetic variants associated with human metabolic disease; Establishment of cell biological processes underlying the association between metabolic disease, neurodegeneration and cognitive decline; Greater understanding of the interaction between genetic and dietary factors contributing to obesity, neuroinflammation and neurodegenerative disease.

How:
o New Treatment Paradigms: Patients will benefit in the long-term from the development of novel therapies based on the molecular pathways affected by genetic variants associated with metabolic disease and cognitive decline potentially leading to new, more personalised treatment and/or preventative paradigms.
Output 4. Small molecule screen.

How:

o Therapeutic Efficacy: Individuals suffering from obesity-related disease will benefit from the development and eventual clinical use of small molecules identified through zebrafish studies leading to improved outcomes for obesity-related conditions.

o Preventative Strategies: Long-term application of these small molecules might also lead to preventive strategies, reducing the incidence or severity of obesity-related morbidity.

Output 5. Publication of Research Findings :

How:
Influence on Policy and Guidelines
o Clinical Guidelines: Research findings and subsequent publications can influence clinical guidelines and best practices for managing conditions related to obesity and metabolic disease.

o Healthcare Policy: Long-term data from zebrafish studies may inform healthcare policies and funding decisions, emphasizing the importance of early intervention and targeted therapies based on model organism research.
The general public will benefit in the long term from Economic and Social Impact:

How:
o Healthcare Cost Reduction: Effective treatments and preventive measures, initially validated in zebrafish models, can reduce the long-term healthcare costs associated with managing chronic conditions stemming from obesity and metabolic disease.

o Quality of Life: Improved therapies can significantly enhance the quality of life for patients and their families, leading to broader societal benefits.
Overall by using zebrafish as a model organism, the project can provide significant insights and advancements in understanding and treating human diseases, demonstrating the translational potential of findings from model organisms to human health.

How will you look to maximise the outputs of this work?

To maximise outputs, we aim to publish in well renowned journals, publicise findings via social media, university official websites, national Public Engagement events and at national and international conferences. We will collaborate with other researchers in the field, including industry, to ensure realisation of potential therapeutic benefits.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): Adult wild type or genetically modified fish: 2,000, wild-type or genetically modified larvae: 20,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Regulation of energy intake and expenditure involves many organ systems including the brain, intestines, skeletal muscle, and adipose tissue. Therefore, whole animal models are essential for better understanding of the development and progression of metabolic dysfunction. Zebrafish are non- mammalian vertebrates that have been shown to have a translationally relevant behavioural and metabolic repertoire as well as conserved neuroinflammation and neurodegeneration pathways and are an established model to study obesity. Zebrafish have been extensively used as a genetic model for the study of development and thus a large number of resources including transgenic lines and mutants are available. Thus, they are the vertebrates with the lowest neurophysiological sensitivity likely to yield results relevant to the human condition.
Invertebrates such as Drosophila and C. elegans are other popular model systems for genetic analyses. However, despite being useful models for the metabolic and neurodegeneration analysis; these organisms share 60 and 40 percent of the genome with humans while zebrafish share 70%.

Furthermore, invertebrate brains do not show the same level of complexity as vertebrates, and it is not clear whether the neuronal networks established to be involved in appetite homeostasis are present in invertebrates. Thus, findings from Drosophila and C. elegans are less likely to be relevant to human biology than findings from studies on vertebrates such as zebrafish where the conservation of neuronal networks has been established.

We use both larval and adult forms in our studies. Wherever possible, assays are conducted before 5 days post fertilisation (dpf), the age at which zebrafish are considered to be free-feeding and are protected by UK legislation. However, more complex behaviours associated with neurodegeneration and appetite control diseases, satiety measurement, deficits in social interaction, cognitive and memory phenotypes, are not evident until juvenile or adult stages. In addition, many of the environmental factors that impact behaviour operate over an extended period or at juvenile stages requiring the use of older forms.

Typically, what will be done to an animal used in your project?

Animals will be bred, reared in non-standard conditions with non-standard feeding in the presence and absence of test substances.
Specifically,

Non standard rearing conditions: wildtype, heterozygous and homozygous mutant animals will be bred. At 3-5 dpf wild type (AB, TU or Tupfel) or GAA mutant larvae are placed individually in a well of a 48 well multi-well plate in an appropriate amount (e.g.1-2 ml) of fish water or physiological saline, or in groups of 3-5 in a well of a 24 well plate (or similar container) with an appropriate amount of fish water in the presence or absence of a test compound for up to 14 dpf. Larvae are moved from 48 well to 24 well or from 24 well to 6 well plates in an appropriate amount of water as they grow as necessary.
Juveniles and adults may be reared in non-standard conditions with high fat diet for up to 12 weeks.

In the way of assessing neuroinflammation and susceptibility to it, some fish may be reared in non- standard conditions (in multi-well plates) and receive inflammation inducement by exposure to inflammatory agents such as hypoxia, LPS administered through the water or by injection, or high fat diet to assess neuro-inflammation and the behavioural outcomes from that. To evaluate susceptibility to obesity and obesity-related

neuro-inflammation and neuro-degeneration larvae may be reared in non- standard conditions with high-fat dietary supplements for up to 12 weeks as larvae, juvenile and adults. In the majority of cases, fish will only receive one injection/exposure and behaviour assessment during their lifetime. The exception to this may be evaluation of eating behaviour as larvae and subsequent evaluation of a second cognitive behaviour as adult.

Behavioural assays in adults involve performance of operant learning tasks, as well as evaluation of natural behaviours such as response to a novel environment, startle responses, or social interactions. Larval behavioural assays are restricted to assessment of natural behaviours such as, response to a novel environment, startle response, food intake, or locomotion. Wildtype and genetically mutant animals will be assessed for feeding and cognitive behaviours at larval and/or adult stages.

Animals will be assessed for performance in behavioural assays. Depending on the assay, behaviour may be assessed over a period of 5 min (response to novelty, social interaction), 1 hr (reward response to food or drugs of abuse) or up to 2 hours a day over a couple of weeks (operant learning tasks and satiety measurement in adults). Animals may be singly housed for up to 1 week prior to behavioural analysi and during the time course of the assay.

We assess the interaction between genetic variants and different dietary parameters and the effects on developmental and immunological responses using biochemical measurement of tissue samples.

What are the expected impacts and/or adverse effects for the animals during your project?

No adverse effects are expected from being reared in Petri dishes or the behavioural assays themselves.

Animals used in high fat diet experiments may show excessive weight gain. Where animals show weight gain that affects locomotion, swimming patterns, breathing patterns, fish will be removed from the study and killed by schedule 1 method.
Animals exposed to neuroinflammation-inducing agents may show signs of neural toxicity (abnormal behaviour, difficulty swimming or breathing). Larvae showing signs of harm will be removed from the study and tissue collected for biochemical and cell biological analysis. Exposure to neuro- inflammatory agents may lead to altered behaviour such as poor cognitive performance or increased anxiety in behavioural assays. This is an outcome measure determined at the end of the assay : duration ranging from 5 min for assessment of anxiety-like behaviour to up to 3 weeks for evaluation of cognitive performance. Animals showing increased anxiety evident as rapid breathing and altered swimming behaviour and failure to habituate to the behavioural assay equipment over a 10 min period on any one day will be removed from the study that day. Any animal that fails to habituate to the apparatus repeatedly over 3 days are not used further for behavioural studies but may be used for breeding or tissue-based studies.

We predominantly assess behaviour in existing mutant lines that have no obvious morphological defects or clinical impairments. No animal showing abnormal development other than tendency for weight gain will be reared.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

69% of animals will be exposed to protocols of mild severity

16% be exposed to moderate severity (those used in inflammation studies or studies of reward or moderate breeding protocol).

15% are expected to be exposed to protocols of sub-threshold severity (those used for breeding purposes only or as wild type controls in adult energy metabolism protocol).

What will happen to animals used in this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

It is not possible to study metabolic processes that involves the coordination of different organs or to assess complex behaviours due to neurodegenerative disease without the use of animals.

It is not possible to test causality using human subjects, nor to conduct cell biological studies to determine cellular processes affected. Even though using cells could help to understand the metabolic pathways, in vitro essays exclude the interaction within a whole complex organism. It is not possible to study effects of obesity on cognition without recourse to animals studies.

Invertebrates such as Drosophila and C.elegans are other popular model systems for neurodegeneration and metabolic studies that have been used to replace the use of vertebrates. However, despite being useful models for the metabolic and neurodegeneration analysis; these organisms share only 60 and 40 percent of the genome with humans while zebrafish has the next closest to human genome organism with 70%. The latter make more reliable date to be extrapolated to humans. Furthermore, invertebrate brains do not show the same level of complexity as vertebrates, and it is not clear whether the neuronal networks established to be involved in human behavioral disease are present in invertebrates. Thus, findings from Drosophila and C. elegans are less likely to be relevant to human biology than findings from studies on vertebrates such as zebrafish where the conservation of neuronal networks has been established.

Which non-animal alternatives did you consider for use in this project?

We collaborate with human geneticists and clinicians using patient derived cell and tissue cultures as well as mouse embryonic fibroblasts to examine the role of genetic variants associated with obesity and to identify potential therapeutics.
We considered experiments in mouse embryonic stem cells (MEFS), patient derived cell and tissue culture and organ on a chip approaches.

Why were they not suitable?

Although human studies can identify genetic variants associated with disease phenotypes, the massive variety of human diet coupled with variance within human cohorts makes identification of significant associations difficult. Further, as mentioned above, it is not possible to test causality using human subjects, nor to conduct cell biological studies to determine cellular processes affected. Even though using cells could help to understand the metabolic pathways, in vitro essays exclude the interaction within a whole complex organism and it is not possible to study effects of obesity on cognition without recourse to animals studies.

Although we and our collaborators have gained valuable insight into metabolic processing from cells in culture, these cells do not recapitulate the full spectrum of the obesity-related disease seen in humans. For example, the majority of tissue culture studies utilise a single cell type and cannot give us information regarding the possible interactions in the complex environment of a developing or adult body that results from different cell types within a tissue or developing animal. We will continue to use tissue culture as the first step in our search for therapeutics.

We have also explored the possible use of organ chip technologies. However, although current organ chips can model some individual organs and in some cases multi organ systems, systemic models, as necessary to establish the in vivo and developmental function of genetic variants associated with obesity and obesity-related morbidity and identify potential therapeutics, are not yet developed.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Numbers are based on previous studies in our lab and the lab of others in the field.
20 adult fish (10 breeding pairs) are maintained for each line with a new generation bred every 6 months. 10 lines of 20 fish bred 2x per year is 400 fish per line over the course of the project. 400x5 is 2000.
For biochemical analysis at larval (4-10 dpf) time points, studies of cell extracts suggest 5-10 mg of tissue is required per replicate. This amount of tissue can be obtained from approx 20-30 4-7dpf larvae (Kirla et al, 2018). For 3 replicates per treatment and 2

treatments per experiment (e.g. with and without drug exposure) and 2 genotypes (wildtype or homozygous) 30x3x2x2 = 360 larvae are required.
Kirla et al (2018) :_Front. Pharmacol. Sec. Predictive Toxicology Volume 9 - 2018 | https://doi.org/10.3389/fphar.2018.00414
For behavioural analyses numbers are based on previous studies in our lab and the lab of others in the field.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use research experimental tools like the NC3Rs Experimental Design Assistant ensure appropriate study design and to help reduce the number of animals used. Wherever possible we conduct studies in cells in culture before experiments in animals.
To reduce the number of animals used we have established a collaboration with other large zebrafish institutes in the UK and the USA, whereby they provide adults or embryos from their mutagenesis screens and transgenesis programs thus reducing the number of new mutagenized/ transgenic animals generated.
WHever possble we obtain tissues samples from animals used in behavioural analysis to reduce the number of animals used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

To minimise number of animals used we will optimise genotyping protocols so as to allow genotyping at early life stages to increase breeding efficiency. Pilot studies will also be undertaken for all new studies to help minimise animal usage. Wherever possible animals that are to be killed following experimental or behavioural analysis will be used to provide tissue for molecular studies and tissue will be shared. Wherever possible we will conduct multiple developmental and/or biochemical analyses from all animal tissue obtained.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use zebrafish as our model species. Zebrafish are non-mammalian vertebrates that have been shown to have a translationally relevant behavioural repertoire and conserved drug reward response and good similarity in the brain regions functionality. Thus they are the vertebrates with the lowest neurophysiological sensitivity likely to yield results relevant to the human condition. Zebrafish have been extensively used as a model for the study of development and thus a large number of resources including transgenic lines and mutants are available. Zebrafish embryos and larvae are transparent allowing visualization of morphological defects at early life stages minimising harm.

We primarily use observation of natural behaviours or food intake as our means of assessing behavioural phenotypes. Behavioural and metabolic assays of natural behaviours are not predicted to cause pain, suffering or lasting distress or harm. Metabolic changes such as overeating and fasting can be considered stressors less severe than those using aversive learning. Although on occasion we induce neuro-inflammation in fish (using for example LPS in neonates or high fat diet at later stages, these are generally mild and short-lived, using anesthetic to avoid suffering.

Why can’t you use animals that are less sentient?

Invertebrates such as Drosophila and C.elegans are other popular model systems for behavioural and metabolic genetic studies. However, despite being useful models for the analysis of neuroinflammation and food intake, the translational relevance of assays for metabolic and neurodegenerative disease phenotypes (cognition) have yet to be established in these species.

Furthermore, invertebrate brains do not show the same level of complexity as vertebrates, and it is not clear whether the neuronal networks established to be involved in feeding and cognitive behaviours are conserved. Invertebrate organisms' genomes show less homology with humans while zebrafish has the closest homology to human after mammals making zebrafish a more reliable model to be extrapolated to humans. Thus, findings from Drosophila and C. elegans are less likely to be relevant to human biology than findings from studies on vertebrates such as zebrafish where the conservation of neuronal networks has been established.

We use both larval and adult forms in our studies. Wherever possible, behavioural assays are conducted at larval stages. However, more complex behaviours associated with neurodegenerative and metabolic disease, such as poor impulse control, deficits in social interaction, cognitive decline, are not evident until juvenile or adult stages. In addition, many of the environmental factors that impact behaviour operate over an extended period or at juvenile stages requiring the use of older forms.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Refinements to minimise suffering include conducting analyses at larval stages wherever possible, increased monitoring for all experimental animals, particularly following any invasive procedure (skin swabbing/ anaethesia/ injection, exposure to neuroinflammatory agent) or drug exposure. We regularly review protocols and the use of analgesics.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow FELASA and LASA best practice guidelines (e.g. see Alestrom et al 2020) and the ARRIVE guidelines. In addition, we are members of the EU zebrafish society (https://www.ezsociety.org/), a society dedicated to promoting and improving the use of zebrafish for biomedical research. Through active participation in this society, we are party to EU wide best practice advice regarding the use of zebrafish for research (see Alestrom et al 2020).

Aleström P, D'Angelo L, Midtlyng PJ, et al. Zebrafish: Housing and husbandry recommendations. Lab Anim. 2020;54(3):213-224. doi:10.1177/0023677219869037

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are kept up to date with advances in the 3RS through the NC3Rs newsletter direct to personal email addresses and via Institutional notification from the NACWO, compliance officer and NVS, and through the AWERB.

88. Validating predictive computer models of temporal changes in the masticatory system

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Key words

Bone, Muscle, Biomechanics, Computer modelling

Animal types	Life stages
Rabbits	Adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To conduct a comprehensive validation of a computer model that can predict musculoskeletal adaptations, and thus replicate (i.e. reduce, refine and/or replace) invasive animal experiments. The data required for such a validation do not exist for the masticatory system of any experimental animal. Therefore, a systematic anatomical and biomechanical investigation is required in which all the primary model inputs, measures of feeding mechanics and temporal adaptations of the musculoskeletal system are quantified and used as a benchmark to measure model accuracy and 3Rs potential.
Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This outputs from this project will not only help researchers understand how the musculoskeletal system adapts to changes to normal function, but will also generate computational models that can be used to replicate biomedical experiments that are frequently performed on animals. Such experiments are performed to test a range of things, such as the effects of disease/injury and the biomedical devices on the musculoskeletal system. These experimentations, like many in musculoskeletal research, can be highly invasive, and cause pain and distress to the animals before they are euthanized. Advances in computational modelling now enable models to predict how the body reacts to dysfunctions to the musculoskeletal system caused by such experiments. Therefore, by replicating biomedical experiments, computational modelling has the potential to reduce or even replace the use of animals in musculoskeletal research and medical device design.

What outputs do you think you will see at the end of this project?

- Scientific publications, conference presentations and a project website to disseminate scientific findings and their 3Rs implications.

- An unprecedented data set (made available through publications and online data repositories) on masticatory biomechanics in the rabbit, and how muscle and bone are modified by surgical intervention.

- A series of freely available, validated biomechanical computer models that can be used in future musculoskeletal research in mammals as an alternative to direct animal experimentations (3Rs).

- A series of systematically derived quantitative measures of computer model accuracy and the sensitivity of model accuracy to anatomical and physiological input data. Together these will provide quantitative guides for the musculoskeletal research community that be used to aid experimental design and minimise animal use in future experiments by replacing specific levels or types of experimentation with predictive computer models.

Who or what will benefit from these outputs, and how?

Short term: The immediate, short-term benefits of this research programme are likely to focus on academics working on rabbits and masticatory biomechanics in general. Our project will generate a unique anatomical and experimental data set on mammalian mastication that will allow us to directly test hypotheses about their unique morpho-functional adaptations to feeding and for diagnosis and treatment of dental disease, which is one of the most common diseases reported with the rabbit.

Intermediate term: Following the publication of our anatomical-experimental data sets, our computational models will benefit a very wide range of researchers. Through the development of the most robust, validated computer models ever constructed, we will facilitate a step change in understanding model accuracy and usefulness and in doing so provide a marker for future biomechanical research. Our model will be utilized by applied researchers interested in understanding both healthy and “abnormal” biomechanics (see above), including anatomists, functional morphologists, palaeontologists, muscle

physiologists, biomechanicists, computer modelers and veterinarians. A major benefit will be our demonstration of the requirements to achieve suitable accuracy in the form a subject-specific model, meaning we will be able to detail which information must be collected in a given animal to ensure the desired level of model accuracy.

Long term: In the long term we hope that our models will demonstrate that computer simulation approaches can contribute significantly to reduction, replacement and refining animal use in biomechanics research, and in surgical and implant design studies that require biomechanical analyses. Experimental studies of this type are highly invasive, and typically cause pain and distress to the animals before they are euthanized. The nature of the approach we will take in this research programme will mean that the potential for future 3Rs benefits in this context will not be restricted to the use of rabbits in dental surgery and implant research. By demonstrating which anatomical and physiological parameters (e.g. muscle size, architecture, contractile properties) need to be measured directly from the species under study to achieve accuracy in a range of biomechanical output parameters (e.g. bone strains, muscle power, joint forces and moments) it will allow researchers working on any vertebrate species to, at the very least, refine their experimental approaches to focus on the key predictive input parameters and reduce experimental measures where species-specific values for less predictive input parameters are not required to achieve sufficient accuracy.

How will you look to maximise the outputs of this work?

This project represents a collaboration between biologists with disparate expertise in motion analysis, bone mechanics and muscle physiology, as well wider collaboration with mechanical engineers carrying out sophisticated computer simulations. The work is funded by a four-year grant that funds two PDRAs split between the two universities, thereby fostering fluid exchange of skills, knowledge and new understanding. This grant also includes funding to support wide dissemination of the new knowledge generated by the project through open access publication, conference presentations, a project website, engagement with industry, impact symposia and public engagement activities.

Species and numbers of animals expected to be used

- Rabbits: 50

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will work on adult, male New Zealand white rabbits as they are the smallest experimental animal of sufficient size to allow all our in vivo measurements to be taken simultaneously during mastication.
Studying male New Zealand white rabbits allows us to reduce the number of animals used in this project, as we already have a full, time-zero, control group data set (N=14) from our previous work.

Rabbits are the first-choice experimental animal for dental implant design and bone growth studies, using highly invasive approaches. Our research will make a scientific contribution to these areas, but also will allow fewer rabbits to be used in future - a direct 3Rs benefit. Further work on rabbit masticatory biomechanics is required to understand the causes and progression of dental disease in rabbits, a major health and welfare issue. Finally, rabbits are generalist masticators, meaning they can serve as a broad model system, making our validation experiments applicable to a wide range of other species.

Typically, what will be done to an animal used in your project?

Cohorts/Overview

Three separate populations of male New Zealand white rabbits will each be split into two groups; control group and intervention group. The intervention group will undergo bilateral myectomy of the ventral portion of the superficial masseter, while the control group will undergo sham surgery. All three populations will be subject to the methods described below, with only the experimental duration for each population varied: Population 1 (N = 7 intervention) will be measured after 1 week post-surgery ; Population 2 (N = 14; 7 intervention and 7 control) will be measured after 7 weeks post-surgery; and Population 3 (N = 14; 7 intervention and 7 control) will be measured after 13 weeks post the initial myectomy or sham surgery. No control group is required in population 1 because we have already collected the necessary data on N=14 rabbits in previous work. The timeframe between populations is necessary to observe gradual changes in kinematics and kinetics, and muscle and bone properties, resulting from adaptation to the muscle myectomy. The animals in Populations 1-3 will be euthanised after the stated timeframe for each population, in order to experimentally measure musculoskeletal adaptations using a combination of micro-CT, MRI, muscle physiology and dissection. The integration of the experimental work below will provide a comprehensive physiological and biomechanical data set that is essential for computational modelling validation and 3Rs analysis.

In-vivo kinematics, kinetics & physiology of mastication

All rabbits in the experimental cohorts (see above) will undergo various measurements to quantify how their bones move and how their muscles generate those movements during mastication. All rabbits in populations 2 and 3 will undergo a second surgery under general anaesthesia to implant tantalum beads in the bones (for x-ray kinematic measurements of skull and jaw bone motion) EMG (to measure muscle activity), sonomicrometry crystals and/or tantalum beads (to measure muscle fascicle length change) and/or strain gauges (to measure bone strain) approximately 1 week prior to the measurements. Note that rabbits in population 1 will undergo the same implantations as populations 2 and 3, but these will occur at the same time as the myectomy. Specifically, sonomicrometry crystals and/or tantalum beads will be implanted into up to three muscles per rabbit and tantalum beads will be inserted into the mandible/cranium. EMG electrodes will also be implanted in the muscles adjacent to the sonomicrometry crystals to allow muscle activity and length change to be quantified simultaneously. Up to 3 strain gauges will be attached to the skull or mandible within a single rabbit, in varying locations to sample strain across the skull/mandible. After recovery (2-5 days), lightweight cables will be attached to the connectors to connect the sonomicrometry crystals to the Sonometrics system and each pair of EMG electrodes and the strain gauge to differential bio-amplifiers. While instrumented in this way, rabbits will be placed in a Perspex cage/box and filmed eating a variety of food types (e.g. pellets, apple, carrots, lettuce, pineapple) using biplanar x-ray

videography. The Perspex box/cage will be filled with saw dust and droppings from the rabbits own habitual cage, and the rabbit will be acclimatized to the box/cage by spending short feeding periods in it during the pre- surgery and post-surgery recovery period. Once this in-vivo data collection on mastication is complete these rabbits will be euthanised using a Schedule 1 killing method so that imaging and dissection can be carried out to quantify hard and soft tissue anatomical properties and to remove bundles of muscle fascicles for subsequent physiological measurements. Nanoindentation will be performed on a cranium of the rabbits in Populations 1-3 to measure the bone material properties (e.g. stiffness) at an unprecedented scale, at a spatial resolution of typically 0.1mm. MRI and manual dissection will be used to carry similar comparisons of muscle architecture between the different populations, and to derive the necessary input data for the computer models.

What are the expected impacts and/or adverse effects for the animals during your project?

The techniques that will be used in this study are well established in the literature and (with the exception of the myectomy) we applied them to rabbits (N = 14) in our previous work. Based on this, we believe that the likelihood of adverse reactions to the experimental interventions to be low because none were encountered in our previous work. Possible problems include:

1) Skin irritation above implantation site: Veterinary advice will be sought for any rabbit showing signs of infection (redness, irritation excessive grooming) and will be treated. Surgery will be carried out in aseptic conditions, and our experience indicates that likelihood of incidence is low.

2) Inappropriate depth of anaesthesia: Potential for inappropriate anaesthetic depth that could result in pain or death during surgery. The likelihood of incidence is low (heart and breathing rates monitored throughout surgery).

3) Intra-operative blood loss: Intra-operative haemorrhage due to damage to blood vessel(s) during surgery. Our surgical experience and experience in the techniques employed indicates that the likelihood of incidence is low.

4) Post-Operative Pain: Some discomfort may be experienced from the implantation of muscle transducers and strain gauges, the myectomy of the ventral portion of the superficial masseter muscle and from the surgery generally for up to 24 hours. Surgery will be carried out under appropriate general anaesthesia with perioperative analgesia, all in accordance with the advice of the NVS. Postoperative pain will be controlled by analgesia. Our experience indicates that implantation of muscle transducers and strain gauges, and the surgery generally does not interfere with the normal behaviour, including mastication, of the rabbit and the likelihood of incidence is low. We have chosen a partial myectomy (ventral portion) of the superficial masseter so that post-recovery the animals we remain able to feed on their full range of normal food types, but with some alteration of the muscle recruitment and jaw motions used to do so. Animals will only be permitted to continue in experiments if they are moving freely and showing no signs of illness and/or pain such as listless or inactive behaviour, stopping eating or drinking, huddled posture, lameness, hyper ventilation, closed eyes, or abnormal droppings. Veterinary advice will be sought for any rabbit giving cause for concern and additional analgesia may be given if appropriate. If

a rabbit fails to recover it will be killed by a schedule 1 method and used for anatomical analyses.

5) The connectors from the wires from the crystals, electrodes and gauges will be left exposed outside the skin but secured with bandaging/ tape so they do not interfere with the rabbits (e.g. during feeding) during the surgery recovery period. However, it is theoretically possible that rabbits could interfere with the connectors and cause minor skin/tissue damage. Our surgical experience and experience in the techniques employed indicates that the likelihood of incidence is low, but we will monitor the connectors and surrounding skin for signs of damage and irritation.

6) Stress during experimental data collection: There is potential for animals to experience stress during the recordings of in vivo kinematics and kinetics due to unfamiliar surroundings. Stress will be minimized by prior short periods of acclimatization to the Perspex box (e.g. 30-60 minute sessions, 1-2 times per day both pre- and post-surgery) during which time the rabbit will be given food treats to hopefully encourage rapid feeding thus reducing time needed for imaging. During data collection, the Perspex enclosure will be filled with saw dust and droppings from the rabbits own habitual cage and water will be provided.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Moderate for 100% of animals in both control and intervention groups.

What will happen to animals used in this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

A comprehensive validation of a computer model that can predict musculoskeletal adaptations, and thus replicate invasive animal experiments, requires detailed in vivo and in vitro animal experimentation to: (1) measure a range of musculoskeletal variables to determine temporal adaptations during the form of experimental testing that the modelling aims to reduce or replace; (2) directly measure a high proportion of the computational model inputs so that the model represents the anatomy and behaviour of a real animal to a high degree; (3) enable the accuracy of the model predictions to be assessed by comparing them to directly measured, real data from the same animal as the model inputs. These data do not exist for the masticatory system of any experimental animal. Therefore, a systematic anatomical and biomechanical investigation is required in which all the primary determinants, measures of feeding mechanics and temporal adaptations of the musculoskeletal system are quantified.

Which non-animal alternatives did you consider for use in this project?

We do not consider there to be any viable non-animal alternatives. The goal of the project is to test the validity of a potential non-animal alternative (computer models).

Why were they not suitable?

The goal of the project is to test the validity of a potential non-animal alternative (computer models). Animal data is therefore required to quantify validity.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Based on previous, similar work on the masticatory system of rabbits, we estimate that 10 individuals will be required to yield 7 high-quality data sets per group.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will work on adult, male New Zealand white rabbits as they are the smallest experimental animal of sufficient size to allow all our in vivo measurements to be taken simultaneously during mastication.
Studying male New Zealand white rabbits allows us to reduce the number of animals used in this

project, as we already have a full, control group data set (N=14) for our Time 0 condition from our previous work.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will work on adult, male New Zealand white rabbits as they are the smallest experimental animal of sufficient size to allow all our in vivo measurements to be taken simultaneously during mastication.

Studying male New Zealand white rabbits allows us to reduce the number of animals used in this project, as we already have a full, control group data set (N=14) for our Time 0 condition from our previous work. During the study we will continuously use power calculations to review the number of individuals being used, based on the actual changes measured, to ensure that we use the minimum number of animals possible without compromising the reliability of our study. . Also, it has been shown that growth rates vary in males and female rabbits and hormonal differences between the sexes are known to exist that impact on bone remodelling (Tunheim et al. 2023). Such hormonal differences

are not represented in biomechanical models, thus the mathematical bases of current models are hormone-independent and attempt to predict bone development based purely on (sex independent) mechanical inputs. Thus we would expect the models to achieve very similar level of accuracy in males vs females rabbits, just as would expect similar levels of accuracy vs input parameter precision in other mammalian species. Studying both males and females would therefore include the naturally disparate rates of bone growth and remodelling and add confounding variables to the analyses. In such instances we would then need to extend the assessment of model accuracy in the same way as adding another species with a different growth rate to male rabbits, and this would essentially form a second, separate validation experiment (i.e. not one where data could be legitimately mixed or considered together, and thus not minimising the number of animals used). Our goal is to demonstrate how well current model algorithms can predict bone remodelling when the majority of anatomical inputs to models are defined with high fidelity, and subsequently how prediction precision is affected when anatomical input accuracy is reduced (mimicking a situation where fewer animals have been used to build the models, which has direct 3Rs implications). Studying two sexes would therefore be analogous to conducting the experiment or addressing this aim twice, with no reason to expect (based on the way the models predict biomechanical function and bone regrowth) any difference in the final result in terms of absolute and relative accuracy (and thus 3R's potential).
Tunheim, Erin Grinde, Hans Erling Skallevold, and Dinesh Rokaya. "Role of hormones in bone remodeling in the craniofacial complex: A review." Journal of Oral Biology and Craniofacial Research (2023).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The goal of this work is to conduct a comprehensive validation of a computer model that can be used to predict musculoskeletal adaptations, and thus replicate (i.e. reduce, refine and/or replace) invasive animal experiments. The data required for such a validation do not exist for the masticatory system of any experimental animal. Therefore, a systematic anatomical and biomechanical investigation is required in which all the primary determinants, measures of feeding mechanics and temporal adaptations of the musculoskeletal system are quantified. We will work on adult, male New Zealand white rabbits as they are the smallest experimental animal of sufficient size to allow all our in vivo measurements to be taken simultaneously during mastication. Studying male New Zealand white rabbits allows us to reduce the number of animals used in this project, as we already have a full, control group data set (N=14) from our previous work.
In order to obtain the necessary kinematic and kinetic data with which to validate our computer model, there is a requirement to surgically implant transducers and strain gauges – i.e. the protocol has a "moderate" severity. Approaches with a "mild" severity

would be unsuitable as the data could not be obtained, hence our approach has the lowest severity possible. Muscle activity can be detected non- invasively using surface electromyography rather than implanting indwelling electrodes as we plan to use. However, surface EMG electrodes are large, they are non-specific and there is much uncertainty in the muscle targeted, they are prone to interference from adjacent muscles and can only be used to record from superficial muscles. While the approach is non-invasive, the method is wholly unsuitable. Muscle length changes have been detected in humans using ultrasound. However, whilst this technique is also non-invasive the ultrasound probes are large and would interfere with the feeding behaviour of the rabbit. The data collected via ultrasound are 2D, whereas muscle length changes occur in 3D – i.e. they can be recorded accurately using sonomicrometry or by tracking tantalum beads but not when using ultrasound. We are unaware of non-invasive techniques that could be used to record bone strain. The techniques that will be used in this study are well established in the literature and extensively applied to rabbits in our previous work without complications. Based on this, and the work conducted in our previous pilot experiments, I believe that the likelihood of adverse reactions to the experimental interventions to be low.

Why can't you use animals that are less sentient?

We will work on adult, male New Zealand white rabbits as they are the smallest experimental animal of sufficient size to allow all our in vivo measurements to be taken simultaneously during mastication.
Rabbits are the first-choice experimental animal for dental implant design and bone growth studies, using highly invasive approaches. Our research will make a scientific contribution to these areas, but also will allow fewer rabbits to be used in future - a direct 3Rs benefit. Further work on rabbit masticatory biomechanics is required to understand the causes and progression of dental disease in rabbits, a major health and welfare issue. Finally, rabbits are generalist masticators: meaning they can serve as a broad model system, making our validation experiments applicable to a wide range of other species. Studying male New Zealand white rabbits allows us to reduce the number of animals used in this project, as we already have a full, control group data set (N=14) from our previous work.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Rabbits will be given a two-week acclimatisation period, during which time they will be acclimatised with the Perspex cage/box that will be used during biplanar x-ray videography feeding experiments. Surgery will be carried out in aseptic conditions, using sterilized instruments and proper techniques within the BSU surgical suite. Skin is sterilized before surgery. A heat source will be provided to maintain body temperature during surgery. Surgery will be carried out under appropriate general anaesthesia with perioperative analgesia, all in accordance with the advice of the NVS. Postoperative pain will be controlled by analgesia. Animals will only be permitted to continue in experiments if they are moving freely and showing no signs of illness and/or pain such as listless or inactive behaviour, stopping eating or drinking, huddled posture, lameness, hyper ventilation, closed eyes, or abnormal droppings. Veterinary advice will be sought for any rabbit giving cause for concern and additional analgesia may be given if appropriate. The rabbit will be given a minimum 48 hour recovery period post surgery prior to data collection, during which time they will continue to be acclimatised with the Perspex cage/box that will be used during biplanar x-ray videography feeding experiments. Veterinary advice will be

sought for any rabbit showing signs of infection (redness, irritation excessive grooming) and will be treated.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will continue review our statistical power as the experiment progresses.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will attend seminars run by NC3Rs and contribute where appropriate as results are generated from this project.

89. Investigating physiological and molecular ageing in mice

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Ageing, Physiology, Phenotyping, Molecular profiling

Animal types	Life stages
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult, Aged animal

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to create a foundational dataset which combines measures of functional decline across the lifespan with corresponding measures of cellular and molecular changes. This will improve our understanding of the similarities and differences between mouse and human ageing, facilitating the translation of treatments for age-related disability and diseases from pre-clinical mouse models to humans.
Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Ageing is a complex biological process characterised by a gradual decline in physiological function and an increased susceptibility to age-related diseases. By the year 2030, approximately one in six people worldwide will be over the age of 60. This demographic shift will result in the population of individuals aged 60 years and above increasing from 1 billion in 2020 to 1.4 billion in 2030. Looking ahead to 2050, the global population of people aged 60 years and older is projected to double, reaching a significant figure of 2.1 billion. This signifies a remarkable increase in the older population, and whilst these additional years present significant opportunity for individuals, a major determinant of the quality of these additional years is health.
A recent report has analysed current health trends in older people in England and made projections regarding the health outcomes we might expect in those additional years of life afforded by the increase in life expectancy. The projections show a relatively modest change in the overall size of England's population over the next two decades (3% increase per decade). However, nearly all population growth will be among people aged 70 years and older. Consequently, the number of people living with major illnesses is expected to grow by 2.5 million, from 6.7 million in 2019 to 9.1 million by 2040. This means that the extra years gained due to increasing life expectancy will not necessarily be years spent in good health and will add an enormous financial and logistical burden on the health and care services.

As the number of people living with major illnesses and multiple conditions increases, a crucial challenge for ageing research is to understand the molecular and cellular determinants of healthy ageing in order to reduce health inequalities, identify markers of resilience and healthy ageing, and ultimately improve quality of life in later years. Preventing age-related disease with new scientific data and approaches is an achievable goal that would circumvent the financial, personal and societal toll of an unhealthy aged population.

What outputs do you think you will see at the end of this project?

Work conducted under this project licence will generate data and new knowledge that will help advance our understanding of how mice age, and what changes, at the level of the molecules and cells, occur over time resulting in age-related decline in physiological function and the development of age-related diseases. We aim to generate new mouse models that will investigate how altering the function of specific molecules in the cells affects the ageing process. We will compare our data in the mouse to available data in humans to look for similarities and differences between mice and humans to accelerate translation of our findings. Our work will help advance knowledge of processes involved in healthy and unhealthy ageing and the factors which influence the development of age-related diseases and identify potential new drugs or druggable targets. We also aim to identify new biomarkers of ageing (e.g., proteins or DNA in the blood that are released from damaged tissues) that we could use to monitor how well a person is ageing.
Our work will be presented at scientific meetings and published in scientific journals to share the knowledge that we gain with the wider scientific community. Datasets will be shared within the data platform associated with our project network as well as being placed in the appropriate open access data repositories upon publication. We will also share the models that we generate. This will help to advance knowledge in the field for the ultimate benefit of society worldwide.

Who or what will benefit from these outputs, and how?

In the short term, scientists in both academia and industry will benefit from the mouse models and discoveries generated under this programme of work. This will be due to the development of new research tools or models, experimental approaches, or identification of new pathways which, when targeted, yield therapeutic benefit.
The long-term aim is to benefit the wider population through the development of new biomarkers and new treatment strategies and lifestyle interventions to promote healthy ageing.

How will you look to maximise the outputs of this work?

By presenting our discoveries at national and international scientific meetings, publishing our research discoveries, and through collaborations with academics or the pharmaceutical industry, we will be able to maximise the impact of knowledge gained under this programme of work. We will also publish results where the mouse model does not recapitulate the human condition, thereby preventing unnecessary repetition of experiments.
Wherever possible we collaborate with others to share tissue samples, cell or mouse lines, and provide training in methods through collaborative research or participation in workshops.

Species and numbers of animals expected to be used

- Mice: 3000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

In order to fully understand the ageing process, we need to perform experiments in wildtype ageing mice and genetically altered mice across the lifespan, from birth to old age. Therefore, we will use animals across the life stages- neonatal, juvenile, adult and aged mice.
To study the biology of ageing and understand how a specific protein affects the ageing process we need to use genetically modified mice which do not express that protein or express modified (or mutant) forms of the protein that is found in ageing individuals. We can genetically modify the mouse genome to generate mammalian models in which to study the specific molecular changes associated with increasing age. We need to do these experiments in whole animals as ageing affects multiple organ systems within the body, and we need to be able to investigate the interplay between the different organs. There are currently no human or mouse cell models (in vitro models) capable of a comprehensive study of ageing across multiple, interacting physiological systems. We cannot use less-sentient animals such as insects or fish as their nervous system does not work in the same way and they lack some of the immune cells present in humans, meaning that they cannot model the processes that we are interested in accurately. Additionally, the highly sophisticated research tools/reagents required to investigate mechanisms of ageing and translate directly to higher mammals are established. Mice remain the model of choice due to the wealth of the genomics information available, their relevance to humans, and the

relative ease of generating, establishing and cryopreserving mouse colonies. Moreover, well-developed and characterised inbred strains of mice present an opportunity for reducing variability and therefore enhancing reproducibility.

Typically, what will be done to an animal used in your project?

A proportion of the mice on this licence will be used only for breeding. This is due to the need to obtain a suitably sized cohort with the correct genetic alterations that can be studied. New models generated over the course of this project will be preserved, archived and be made available for use by other researchers.
Initial characterisation of our ageing models (standard laboratory strains of mice and genetically altered mice) will include a series of phenotyping tests to characterise their physiological function as they age. For example, mice will be assessed using home cage analysis for behaviour, motor and metabolic parameters, mice will also be tested outside the cage to look at other behaviour and motor phenotypes, they will have their sensory system assessed to check for hearing and sight issues, and blood taken for biochemical analysis. These tests will be used to detect phenotypes that reflect human age-related features seen in the clinic. We will use multiple cohorts of mice. Some will be undergo a set of phenotyping tests and be sampled at defined times points and others will undergo a set of tests that will be repeated at multiple time points across the animal's lifespan up to 26 months of age.

Experiments will vary in duration from days to months. All protocols will end by 26 months of age. Animals that are alive over 52 weeks (i.e., 12 months old) will have increased welfare checks to look for age-related phenotypic changes.

What are the expected impacts and/or adverse effects for the animals during your project?

Ageing mice may develop symptoms such as weight loss, muscle weakness, hearing loss, gait changes, cataracts, tumours and dermatitis. We expect these symptoms will develop very slowly over the lifetime of the mouse. We will not allow these mice to age beyond 26 months and strict humane end points will be adhered to, for example, mice with tumours or dermatitis that are not healing, will be killed.

In a subset of strains, we will be breeding the genetic alteration for the first time. It is possible that this will affect the viability of those mice in the period between birth and weaning. These mice may develop signs of accelerated ageing such as those describe above for normal ageing mice, but these will be seen at earlier timepoints.
Some of the tests that we perform will require the mouse to be under anaesthesia. Repeated anaesthesia is not thought to cause adverse effects, other than the mice may develop an aversion to it. No animal will undergo more than 8 general anaesthetics in its lifetime.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Sub-threshold - 20% of all animals used in this project Mild - 10% of all animals used in this project Moderate - 70% of all animals used in this project
What will happen to animals used in this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animal models are required for the study of ageing because it is a highly complex process, affecting multiple organ systems within the body. For the majority of mouse models we use, we will be studying multiple systems involving many organs of the body, and the interplay between them (e.g., behaviour and metabolism). There is currently no human or mouse cell model (in vitro model) capable of a comprehensive study of ageing across multiple, interacting physiological systems.
We have established a strong international network of collaborators over the years and have access to a rich source of information across many different fields in science as well as in husbandry and welfare. This network can be canvassed to explore areas of research that lie outside of our own expertise, to ensure that we are able to identify areas of work where either the mouse model or the phenotyping test is not appropriate to the scientific question.

Which non-animal alternatives did you consider for use in this project?

Wherever possible, we will use human tissue/cells or cell culture systems to replace animal models of ageing. We have accumulated archival tissue banks of frozen and formalin fixed tissues from our previous models and from healthy and diseased human tissues. These samples are used in multiple ongoing projects to minimise the number of animal models used.

We routinely use human cells in culture to understand and model the biological processes affected by ageing and to perform initial drug testing prior to performing work in animals. We can also use special cell systems called induced pluripotent stem cells to make particular types of cells, such as muscle or brain cells, and investigate the effect of ageing in those cells in culture.

We have also started developing 3D culture systems as an alternative method for understanding ageing mechanisms and drug screening, for example we can grow 3D intestinal cultures. We are also using co-cultures of human neurons, adipocytes and inflammatory cells to model how molecular mechanisms associated with ageing affect the interplay between different cell types. The use of these cell systems prior to work in animals means that we can refine the work we do in animals and minimise animal use.

Why were they not suitable?

Whilst they are useful tools, there are limitations to cell cultures systems, these include:

1. Cells grown in culture need a plentiful supply of nutrients, particularly glucose. This means that they are often not reliant on the same metabolic processes as they are in the body, and if they have molecular changes associated with ageing they may not behave in the same way that they do in the body. These abnormal behaviours could lead to the identification of non-relevant pathways or fail to predict drugs that are likely to be ineffective in modulating the ageing process.

2. Cells grown in petri-dishes sit on plastic, which changes their biological characteristics and they become "super sensitive" or fail to recapitulate their normal biological role. We may develop organoids in future studies once we have generated data from live mice. We will need to compare the data from the two systems to see if we can use organoids in place of mice in future studies.

3. The in vitro screen and systems we use prior to animal studies are extremely useful for aiding our basic knowledge and for initial drug screening, and we use these systems to reduce animal use however they cannot fully recapitulate the behaviour of a drug in a complex biological system

4. Whole-body work is needed to understand the ageing process as there is evidence for the interplay of cells between different tissue/organ systems. For example, it has been shown that cell culture and brain organoid models do not recapitulate the types of brain changes observed in human ageing due to a lack of immune cells, which are cells necessary to drive age-related neurodegeneration, thus limiting their use in pre-clinical investigation. As organoid systems become more advanced, this may become possible, but technology is currently lacking for these applications.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The phenotyping pipeline has been designed in collaboration with many international, renowned mouse centres and has involved the input of many laboratory animal experts. By utilising this pipeline, we maximise the data obtained from each cohort of mice. Combining tests in the same mice allows the interpretation of data to be correlated directly, rather than inferred. This allows us to carry out more advanced statistical analyses to detect correlated trends between assays. Directly correlated effects have a greater sensitivity as the major source of variance (between mice) is removed, opening the possibility to detect subtle effects resulting from gene deletion, thus resulting in more data from fewer mice.

We have used knowledge from previous studies to mathematically calculate the minimum number of animals needed in each group to generate data which allows us to answer our scientific questions. By doing this we can minimise use but be confident that the differences in a scientific measurement between two groups is meaningful and has not been obtained by chance. Group sizes will be larger when we need to keep mice into old

age (up to 26 months). This is because, in our experience there is significant attrition (especially in males) when co-housing animals to this time point. Attrition rates are different for different background strains, but we will use data from other ageing projects to ensure that the appropriate numbers for statistically and biologically relevant data will be available throughout the study.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

NC3Rs guidelines will be referenced during the planning phase of experiments; this includes thorough examination of the literature, evaluation of non-animal alternatives, use of pilot studies, prevention of observer bias (by using standard operating procedures), ensuring sufficient staffing and competency is in place to conduct the selected tests, and deciding on which characteristics of the animal are essential to each specific study. Standard Operating Procedures have been written and used routinely for previous projects. This standardises the way the data and metadata are collected and reduces the variability and therefore the sample size.

In addition, constant evaluation of procedures and refinements will minimise the number of animals used in this project. Careful planning of colony expansion will ensure any surplus of animals generated is kept to a minimum.

To determine the number of animals we will use in the project, we have analysed available phenotyping data across 8 centres world-wide and used power calculations to ensure we are using the minimum number of mice for to obtain scientifically meaningful results. Wherever possible, blinding will be used to avoid introducing user bias, and mice will be pseudo-randomized at weaning to ensure an equal distribution by body mass within separate ageing sub-cohorts (each culled at a different age), as early-life body mass is known to affect lifespan and health span. Sexes are balanced to ensure equal representation, as each sex is known to age differently.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Statistical analysis is performed to determine the minimum numbers of animals needed to generate biologically meaningful data. If this is not possible then pilot studies are performed to reduce numbers and inform future studies going forward.
All breeding for experimental cohorts is performed according to precise breeding calculations which have been designed in consultation with the wider team who have extensive experience with breeding of genetically altered mice. We will continually review data from our breeding colonies and refine and update the breeding strategy if required. This is to ensure we produce the maximum number of animals that can be used in experimental procedures and minimise the number of mice which have the incorrect genotype.

Where breeding information is not known (i.e., for new lines) a small pilot breed will be carried out first to assess welfare and viability of mice with a particular genetic alteration. In addition, we will work with our colony management team and follow local guidelines to preserve colony health, monitor any unexpected genetic defects and ensure preservation of the genetically altered lines.

All new GA lines that we generate and characterise will be shared with the wider research community upon request, via frozen sperm or embryos, depending on the strain.
Tissue is regularly shared between projects to maximise outputs from animal procedures and minimise numbers of animals used. The group have accumulated archival tissue banks of frozen and formalin fixed tissues from our previous models and these samples are used in multiple on-going projects to minimise the number of animal models used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

All of the models chosen are the lowest severity model that can be used to answer our research questions. Ageing affects multiple organs of the body; therefore to understand the molecular and physiological mechanisms of ageing we need to use both normal ageing mice and genetically altered mice in which we have altered specific genes which we know affect the ageing process. As we are interested in mechanisms of ageing, we have to allow the mice to get old. We will continuously monitor the behaviour and condition of the mice, using non- or minimally-invasive tests wherever possible to ensure minimal suffering. Where a mouse is undergoing multiple tests, these will be done in a specific order to limit cumulative suffering and allow the mice to recover from one test before they do the next test. Pain relief and anaesthesia will be given when required to limit pain, suffering and distress.

Why can’t you use animals that are less sentient?

The mouse is the lowest mammalian species (in terms of the sensitivity of the nervous system) in which we can perform the full range of genetic manipulations that we need in order to understand how ageing affects multiple organ systems, the clinical phenotype of the mouse, and how we might modulate the ageing process with therapies or lifestyle interventions. It is possible to carry out some manipulations in frogs and fish but the processes being studied here are in a mammalian context and, although other animal and non-animal species can be informative in this regard, they cannot replace studies specifically in mammals. For example, ageing is known to have an inflammatory component. Fish and insects (e.g., flies) lack the same broad range of immune cells found in humans, which means that there are differences between fish and mammals that could affect the biology. Some genes we want to model are not the same between these species and mammals therefore some of the mechanisms may not be the same. In addition, drugs that target those cells or pathways may not work in these systems due to fundamental differences in the biology of the species compared to humans.
Mice remain the model of choice due to the wealth of the genomics information available, their relevance to humans and the relative ease of generating, establishing and cryopreserving mouse colonies. Moreover, well-developed and characterised inbred

strains of mice present an opportunity for reducing variation and therefore enhancing reproducibility.
The life stages used under each protocol have been carefully considered and meet the experimental life stage needed. For example, we would like to know if phenotypes observed early in life can predict how well an animal ages, therefore tests will be done at different time-points across the whole lifespan and will include both very young mice and aged mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For all tests mice are only housed in modified cages or arenas for the minimum time needed to gather meaningful data. Mice undergoing phenotyping tests are monitored more frequently and are removed from tests if they appear to be suffering from an adverse stress reaction, or other unexpected adverse effects of the phenotyping tests. Mice which have had anaesthesia have extra monitoring until fully recovered and extra checks when back in the holding rooms. When general anaesthetics are necessary, the combinations with least adverse effects will be used, for example for all tests inhalation anaesthetics will be used, with the exceptions of the Auditory Brainstem Response, the Electroretinography and the Optical Coherence Tomography tests which cannot be carried out with the mouse on a face mask. No individual mouse will undergo more than 8 general anaesthetics over its lifetime. Pain from tail bleeds is reduced by using local anaesthesia. Mice over 12 months of age will have increased welfare checks to look for age-related phenotypic changes.

We have modified non-aversive handling techniques to reduce stress on animals. Pipelines are designed with consideration given to the overall experience of the mouse and the number of types of tests any one animal will go through.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Routes and volumes for administration of substances are taken from NC3Rs guidelines. The animal house has full accreditation. To conform to these standards, we must ensure a high level of quality control on all fronts including husbandry, phenotyping and administrative processes.
Standard operation procedures for most tests have been generated using data and expertise from multiple animal houses.
The requirements of the ARRIVE reporting guidelines will be considered at all times during the planning of the experiments and strictly adhered to when publishing the data.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Team members will attend general 3Rs symposiums in the UK and abroad over the course of this project. From these we may gather information on refined phenotyping techniques or housing and husbandry methods. Moreover, it is our intention to continue to present any techniques that we develop ourselves in posters and papers in the relevant scientific/animal care forums.

More specifically, members of the phenotyping team will attend conferences that focus on all aspects of the project and the pipeline. Any new developments which could impact the studies will be discussed within the team of investigators and action taken if relevant to the development of the phenotyping pipeline.

90. Understanding the nature of pluripotency

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Stem Cells, Transgenesis, Biotechnology, Chimaera

Animal types	Life stages
Sheep	Embryo and egg, Juvenile, Adult, Pregnant adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Pluripotency is the term describing cellular potency, which cells can give rise into any somatic cell lineages including germ line. We will assess the property of pluripotent stem cell using animals.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Pluripotency is the term describing cellular potency that a single cell can differentiate into all somatic linages of our body. Pluripotent stem cell has been used to create transgenic mouse lines by injecting embryonic stem cells into blastocyst stage embryos. After the injection followed by the embryo transfer to surrogate mothers, stem cells join the host embryo development. This creates the chimaeric animals which their organs and tissues consists of both host and injected stem cells. This technology has been well established in mouse and such chimaeric animal is called ‘blastocyst chimaera’. But there were no pluripotent embryonic stem cells available from livestock until recently. In 2021, we successfully established the pluripotent stem cell lines from pig, sheep and cattle. Mice

have been used to understand genetics of humans however, recent studies suggest that the mouse is not always the best animal model to understand human development and disease. We are aiming to develop these livestock stem cells equivalent to mouse ones so that all the biotechnology available in mice can be applicable to livestock species. Livestock embryos share similarities to human in embryogenesis and adult livestock (such as pig and sheep) have similar organ sizes and physiology. Research of livestock do not only benefit agriculture and veterinary medicine but also benefit to understand more related to humans. Once we can produce such chimaera competent stem cells and method, it is relatively easy to create transgenic livestock, which can benefit veterinary and medical research, both in basic and clinical sides. For the long-term perspective, such chimaera production technology can be applicable to create stem cell derived transplantable humanised organs using livestock animals.
Scientifically, the knowledge of such stem cells from livestock species will help with a better understanding of the conserved mechanisms of in vitro pluripotency across mammalian species including human.

What outputs do you think you will see at the end of this project?

The results of this work will be published in academic peer-reviewed journals (Scientific publications).

Who or what will benefit from these outputs, and how?

Chimaeric mouse can be created by injecting mouse embryonic stem cells into blastocyst stage mouse embryos. These are called blastocyst chimaeras. In this case, chimaeras consist of both host cells and injected stem cells. This technique is widely used in mice, but there are no such stem cells are reported other than rodents. We recently reported embryonic stem cell lines established from livestock species (pig, sheep and cattle). Once our livestock stem cells can produce the next generation by creating chimaeric animal, such stem cells will be a very useful tool to create transgenic animals in livestock. Advantage of having such stem cell lines is, we can easily and precisely modify their genome in the dish. Gene editing is achievable at the 1-cell stage embryo using recent development of CRISPR technology. However, gene editing at fertilised egg cannot predict the mosaicisms of the animal caused by gene editing activity continued in cleavage stage. Also genome editing at single nucleotide level is not efficiently achieved using the fertilised egg because another rounds of screening of offspring needed to select correctly modified individuals.

Animals derived from genetically modified stem cells have a lot of benefits for not only basic and clinical research but also industries (both pharmaceutical and agricultural). For example, they can be utilised to create animal models of human diseases and aging (these animals have similar organ size to humans and much longer lifetime than mice), and be made disease resistant and or more tolerant to environmental stress such as heat. Such stem cells and the associated know-how have a high potential as intellectual properties. These can be distributed to the academic society as well as industries through technology transfer office.

How will you look to maximise the outputs of this work?

The results will be published in peer-reviewed journal and via pre-print servers such as bioRxiv to accelerate the dissemination of new knowledge and technologies. Collaboration with colleagues from other institutions will be promoted through scientific conferences.

Species and numbers of animals expected to be used

- Sheep: 105

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We have chosen sheep as representative animals of livestock species to understand the functional properties of embryonic stem cells maintained in the dish using in vivo assays. Sheep share more developmental similarities to human than mouse and IVF allows us to prepare blastocyst stage embryos very efficiently. We can relatively easily obtain ovaries from local slaughterhouse to collect oocytes. We will perform the embryo transfer experiment using the chimaeric blastocyst prepared by IVF. We will use ewes as recipients of chimaeric blastocysts.

Typically, what will be done to an animal used in your project?

Ewes will be oestrus synchronised prior to the embryo transfer of day 6-7 blastocyst. Embryo transfer will be done by laparotomy. Animals will be sedated and intubated to induce general anaesthesia. After the operation (up to 45 min), the animals will be transferred to a recovery room where they are expected to make a rapid and unremarkable recovery from anaesthetic. When we collect foetus, surrogate mother will be killed by Schedule 1 method within 24 days after the embryo transfer. When we continue pregnancies to term to obtain lambs, surrogate mothers will be killed by schedule 1 after weaning. Blood, sperm and other tissues will be collected from the chimaeric lambs and chimaeric lambs will be killed by Schedule 1 method to collect tissues between 5 and 8 months old for further analyses.

What are the expected impacts and/or adverse effects for the animals during your project?

No major adverse effects are associated with oestrus synchronisation of ewes. A mild discomfort can be caused by the introduction of the intravaginal sponge. It is important to lubricate the applicator to avoid rubbing.
Surgical procedures will be carried out aseptically. In the uncommon event of post-operative complications, animals will be killed unless such complications can be remedied promptly and successfully using no more than minor interventions by the opinion from the NVS. In the case of wound dehiscence, uninfected wounds may be reclosed on one occasion within 48 hours of the surgery. Peri- and post-operative analgesia will be provided. Reagents will be administrated as agreed in advance with the NVS. Animals are expected to make a rapid and unremarkable recovery from anaesthetic. Any animals that fail to do so would be killed by a schedule 1 method.

In the unlikely event that animals exhibit signs of pain that cannot be controlled by the use of analgesia, or develop infection that does not respond to antibiotic treatment, they will be killed by Schedule 1 method.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Ewes 100% moderate (which will be used for ET). Lambs 100% mild (for blood collection).

What will happen to animals used in this project?

- Killed
- Rehomed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The study is based on using sheep to assess the developmental function of newly established stem cells. Pluripotent stem cells are unique to mammalian species therefore other vertebrates or non- protected animals (such as invertebrates) cannot be an alternative as they are not relevant.
Blastocyst embryos can be cultured for further a few days in vitro, but they never develop more than early gastrulation stages. Thus it is crucial to test their developmental potential using animals. Sheep will be used in the chimaera study as we test the function of sheep stem cells and IVF to develop the host blastocysts is very efficient.

Which non-animal alternatives did you consider for use in this project?

We considered gastruloid model which is often used in human and mouse studies to mimic gastrulation in the dish using pluripotent stem cells. Gastruloids are obtained by aggregating undifferentiated stem cells and culture them for several days in the dish.

Why were they not suitable?

Gastruloids are stem cell derived gastrulation stage embryo like structures however, they have limitations of their developmental potentials. Gastruloids are mostly caudal part of the embryo (elongated tail-like structure having notochord-like tissue and endoderm like lumen but often observed as a disorganised structure) and lacking anterior structure such as head and heart. They do not develop further than early somitogenesis stage therefore we need to use embryos and animals to develop them.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Number of the sheep was estimated based on the efficiency of previously published chimaera work in mice and also based on the efficiency of known sheep embryo transfer experiment.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We employed NC3R's experimental design guidance and experimental design assistant (EDA) website to plan our experimental design and practical steps. We will use the EDA diagram and report output to support experimental planning with animal users.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will test the blastocyst injection followed by the embryo culture to determine the best procedure of the blastocyst injection (which stage of blastocyst and/or number of the cells to inject) prior to performing embryo transfer experiment. This pilot study will reduce the number of ewes used for the experiments.
The animals used for embryo transfer will be used for sampling by other groups in the same campus. When we collect embryos or foetuses, animals will be killed by schedule 1 method, and others collect samples of their interest postmortem. Typically, the parts of the animals shared with other studies are joints (arthritis) and digestive system (gut content).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use sheep to create chimaeric animals. We will perform the blastocyst injections using IVF embryos. In sheep, we are able to produce IVF embryos very efficiently. Maximum of 40% of IVF embryos reach blastocyst stage in our hands.
We will transfer the chimaeric blastocyst by midline laparotomy. Some experimental reports stated that blastocyst stage embryos can be transferred transvaginally. However, this technique is not very efficient and is not recommended for the experimental embryos. Therefore, embryo transfer will be performed under general anaesthesia in our dedicated

operating theatre at BSU under aseptic conditions. A small midline laparotomy will be performed to exteriorised oviduct and perform embryo transfer using a catheter. The procedure should take around 30 min. Animals are given peri-operative analgesics followed by post-operative analgesics to reduce the pain. During the first 24 hours, animals which have received ET will be group-housed. They will be monitored four times a day to look for the signs of discomfort or pain (using grimace scale, also check postural changes, gait, apathetic, and unwillingness to move, eating and drinking behaviour). After this period, animals will be housed together with those received ET previously, for the remaining period of pregnancy.

Why can’t you use animals that are less sentient?

We will test the function of sheep stem cells. We need to use sheep embryos and ewes to transfer. Therefore, other less sentient animals could not be used. There are no methods to develop the embryo beyond the gastrulation stage in the dish in sheep. Therefore we need to transfer chimaeric embryos to the surrogate mother.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

After the surgery, animals are grouped with others which already have received ET within 24 hours. To minimise the discomfort, animals are given the pain killer during this period and they are monitored four times a day. Animals are always group-housed.
Veterinary intervention will be promptly sought as required and appropriate humane endpoints applied in the unlikely event that adverse effects develop and cannot be controlled. Full records of procedures are undertaken, and daily monitoring and veterinary request will be performed.
The animals used for embryo transfer will be used for sampling of other tissues by other groups in the same campus.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Home Office Code of Practice will be used to ensure that animal care and housing is appropriate. This guidance will be used in conjunction with the advice in the NC3Rs Resource Hub for both housing and handling of animals.
The NC3Rs' Procedure with Care will inform personal licensees of refinements in the conduct of the minor procedures undertaken in this licence.
Home Office and FELASA severity information will be used to ensure the actual severity experienced by animals can be recorded and limits within this licence adhere to.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will keep myself updated through latest publications and conference presentations as well as the NC3Rs website.

91. Immunisation of Rodents

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Antibody, Antibodies, Immunisation, Antigen, Discovery

Animal types	Life stages
Mice	Juvenile, Adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The immune system recognises foreign molecules called antigens. In response, the immune system produces defence molecules called antibodies which have shape which specifically binds to the antigen. The defence cells, called B cells involved have particular genes which enable the cell to make the antibody. In the lab, cells can be made called hybridomas. These are made by fusing together a particular antibody-producing B cell with a harmless cancer cell. The hybridomas divide over and over, producing a large clone of identical cells, all of which produce the required antibody, these are called monoclonal antibodies, specific to one antigen. Monoclonal antibodies can be used in basic and applied research, to develop therapies and to detect disease states.
Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

To produce new antibodies at the behest of our clients that otherwise would not be available. The chances of a project being successful and producing useful antibodies increase from the technical expertise provided by the PPL holder. The antibodies will be developed by the clients into novel therapeutic molecules and biochemical reagents to assist their development from lab to clinic and so improving human health.

What outputs do you think you will see at the end of this project?

The outputs of this project are
- DNA sequences of the antibody genes.
- the delivery of novel antibodies tailored to a client's specific needs.
- the antibody secreting hybridoma cell lines that will be provided to the client whose establishment will allow for the permanent sources of antibody production.
- the knowledge and technical innovations gained during the course of a project.

Who or what will benefit from these outputs, and how?

- The client will receive the DNA sequences of the antibodies at the end of the project.
- The client will receive antibodies at the end of the project.

- The client will receive the antibody secreting hybridoma cell lines at the end of the project. These will represent an unlimited source of monoclonal antibody for the client who will use them in developing novel therapeutic molecules and tools to assist their development from lab to clinic.
- Patients with diseases will benefit from novel therapeutic medicines developed from the antibodies that may also be used to detect disease states and monitor medical treatments.

How will you look to maximise the outputs of this work?

I will disseminate any improvements made in hybridoma or B cell technology by publishing or meeting and collaborating with other antibody service providers. Negative results, i.e. failure of an antigen to provoke an immune response or produce specific antibody secreting hybridoma or B cells will be highlighted in publications, talks, posters and discussions with future clients. Clients will be sent proforma forms asking them if they can inform the PPL holder of any literature, talks or posters that mention research that utilise the antibodies. The antibodies may be further developed by the client for the identification of new therapeutic targets or into medicines for combating human diseases.

Species and numbers of animals expected to be used

- Mice: 450

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice have been used in immunology for over 100 years and their immune systems are now understood down to the cellular and DNA level. Mice are the preferred animal for generating monoclonal antibodies, exact copies of the same antibody that all recognise the same antigen, due to the availability of proven immunisation strategies that do not cause adverse effects, ease of breeding, handling and housing and relatively low amounts of antigen are required. Crucially the mouse cancer cell lines exist that allow the formation of antibody secreting hybridoma cell lines. The complex mouse genes that encode the antibody proteins have been sequenced so the unique gene regions that determine the specificity of the antibody can be rapidly isolated, DNA sequenced and cloned. Juvenile and adult mice from 6 - 15 weeks old will be used as their immune systems are sufficiently developed to mount an immune response but immature enough not to have been challenged with many or any other antigens that may reduce immunisation.

Typically, what will be done to an animal used in your project?

At any stage of the project work below and as required, general or local and/or analgesic agents will be administered.

In protocol 1, a pre-immune blood sample will be taken and the animal will be injected with antigen solution under the skin (subcutaneously SC). Every 2 - 3 weeks (up to 4 times), antigen boosters will be administered by intraperitoneal (the abdominal cavity, IP) injection and a post-immune blood sample will be taken after the second booster to allow evaluation of the immune response. The animals will be left to rest for a period of between 1 to 10.5 months following which it will receive a final antigen boost and up to 4 days' later, it will be sacrificed and the lymphoid tissues where the antibody secreting cells reside are harvested. The time period between the first immunisation and the tissue harvesting will be no more than 1 year.

In protocol 2, an animal is immunised up to 4 times SC with antigen and this will be done up to 3 more times over the next 8 - 21 days. A post-immune blood sample may be taken after the second booster to allow evaluation of the immune response. The animal will receive a final boost of antigen on either day 12 (SC) or 25 (SC or IP). Up to 4 days later, it will be sacrificed and the lymphoid tissues harvested.

What are the expected impacts and/or adverse effects for the animals during your project?

The vast majority of animals will appear slightly subdued and slightly hunched after antigen injection that will resolve in 20% of animals within an hour and would be considered mild severity. The remaining animals will not display any clinical signs 3 hours after antigen administration and would be considered moderate. Animals will be placed on heat mats after injection to alleviate the short-term effects of antigen administration such as hypothermia and closely monitored for an hour. Animals will be monitored hourly for the

next 5 hours and any animals not fully recovered at this point will be humanely killed.
Animals will be checked twice daily on non-procedure days.
Any animals showing adverse clinical signs considered untreatable for any reason by animal welfare staff will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Protocol 1: Mild (20%), Moderate (80%)
Protocol 2: Mild (20%), Moderate (80%)

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Only a live animal with a functioning immune system can produce antibodies with the desired characteristics.

Which non-animal alternatives did you consider for use in this project?

Phage and ribosome display, in silico (computer) modelling methods.

Why were they not suitable?

In vitro (test tube) methods such as phage and ribosome display do not produce full length antibodies with the desired affinity. The composition of the antibodies can be unnatural leading to problems in producing a therapeutic medicine. Popular antigen targets like membrane proteins do not generate antibodies with in vitro methods.
In silico modelling of antibodies is at an early stage of development and cannot yet generate antibodies of sufficient affinity.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Animal numbers are based on experience from studies carried out under previous Project Licences and also consulting with other experts in the field. Up to 12 projects a year over 5 years mainly with mice are envisaged. No control groups are required. An antibody discovery screen requires 1 x106 cells that represents the number of cells isolated from 2 animals. While a strong antigen will produce sufficient responses in all animals within a group, weaker antigens can produce responses only in a proportion of the animals. So to ensure a minimum of 2 sufficiently immunised animals at the end of the project, 4 animals will be used per project. In projects where 2 types of antigen are available, up to 4 mice per format will be immunised in parallel.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

On advice from experts in the field, knowledge of the number of cells required to successfully complete an antibody discovery campaign, published literature, study of similar PPLs' NTS on the Home Office website, the number of animals per antigen immunisation is set at 4. The methods and technologies for isolating and screening antibodies were improved making more efficient use of harvested animal tissues and allowing a reduction from 5 animals on older licences to the current 4 per antigen target. Finally, immunisation for the purpose of raising monoclonal antibodies is a qualitative experiment so no control animals are required, thus keeping animal numbers to a minimum. As mice will be purchased from commercial suppliers as required, no breeding colony will need to be maintained locally.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Animals will be purchased from commercial suppliers as required thus removing the need for a breeding colony. To maximise success and outputs, immunisation strategies will be designed to maximise the number of antibodies discovered per animal. Other experts in the field will also be consulted about animal numbers. To provide enough material for a successful antibody screen, 4 animals are required. The reason is that tissue from 2 animals is the minimum requirement for an antibody screen. However, as weakly antigenic substances may not provoke an immune responses in every animal within a group, 4 immunised animals will on average yield 2 animals with sufficient cells for an antibody screen. Reducing the animal group number below 4 could result in insufficient numbers of immunised animals and cause the project to fail in its objective.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice will be immunised with antigen in order to produce reactive B cells. These cells will be extracted for the purpose of antibody sequencing or making antibody secreting cells (hybridomas). These animals are ideal as they are easy to maintain, have short life spans, are easy to handle and cage, thus reducing stress. They have a long history of use in immunology and so the strategies and routes of administration of antigens have been designed to produce a strong immune response and the high probability of isolating monoclonal antibodies without causing long lasting adverse effects. Two kinds of genetically altered animals may be used for generation of monoclonal antibodies; animals containing humanised antibody genes and knockout animals with a specific gene deleted. The humanised antibody animals yield fully human antibodies that can take advantage of all benefits of modern mouse monoclonal technology. The knockout animals are used when the antigen is naturally present within the animal and interferes with the anti-antigen immune response. Both kinds of genetically altered animal can be used as sources for the discovery of human antibodies for therapeutic use.

Protocol 1 will be used to primarily target the spleen tissue. The relatively long time period of the protocol ensures that high quality, high affinity antibodies are produced. A further benefit is that the immune response can be monitored by the presence of antibodies in the blood. Weak antigens do not produce an immune response in every animal so these non-responders can be removed early in the protocol. Protocol 2 is a shortened time period immunisation that primarily targets the lymph nodes and to a lesser extent the spleen. High quality antibodies are produced but may not be as high affinity as those from Protocol 1 due to the reduced time for affinity maturation, the natural process within the animal whereby antibody secreting cells are selected based on their affinity for antigen, which improves during the immune response. Typically Protocol 1 will be the preferred option to generate antibodies but Protocol 2 is the preferred option for mouse antigens or where a human protein is identical to the mouse version and may not provoke a sufficiently strong immune response in Protocol
1. Protocol 2 will be used when recommended by providers of transgenic mouse strains.

Why can’t you use animals that are less sentient?

The production of monoclonal antibodies requires a functioning immune system and mice are short lived, have rapid generation times and are the smallest, easiest to handle and cage mammals that are available from commercial suppliers. Wild type juvenile animals of 6 - 8 weeks old are the optimum age as the immune system has matured to be fully functional but as the animals are housed under relatively clean, pathogen-free conditions, it is unlikely their immune systems will have been challenged. Transgenic animals may be smaller than wild types and in which case, 10 - 15 week old animals will be used.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Prior to initiating a project, all potential antigens will be assessed during initial discussions and the ethical review to identify molecules that could cause harm. Antigens with known anaphylactic potential such as foreign immunoglobulins will be avoided. Antigens will be sourced from reputable commercial suppliers that will be certified free of microorganisms and contaminating substances. Antigen solutions for administration will be prepared using aseptic technique with sterile physiologically compatible buffers such as saline. Antigen preparation records will be kept on file with a description of the antigen, reconstitution procedure, aliquoting (dividing a large sample into multiple smaller samples) protocol,

method of sterilisation and diluents used. The record will be signed and dated by the responsible scientist. After immunisation, animals will be placed on a heat mat to alleviate any possible hypothermia. Monitoring will continue for the next 5 hours and animals not fully recovered at this point will be humanely killed. Animals will be checked twice daily on non-procedure days. Animals that do not develop an immune response after the second boost on Protocol 1 will be removed from the project and humanely killed. Antigens producing intermediate strength responses will be boosted an extra time to allow the immune response to further mature. The administration routes and strategies have been shown to cause no adverse effects while still producing very strong immune responses that are critical for producing sufficient numbers of independent antibodies of the right class with high affinities and diversity.

Animals on Protocol 2 will be immunised primarily through the subcutaneous route multiple times over a relatively short period of time. To minimise any pain from multiple pin pricks, brief anaesthesia will be administered during the injections together with a dose of an analgesic (buprenorphine) for the recovery period.

Over the course of 3 previous PPLs, the PPL holder has not seen any skin lesions such as ulcers, granulomas and inflammation using Freunds adjuvant, a solution used to carry the antigen via the subcutaneous route in a strain of mouse called BALB/C. However, occasional skin lesions were observed with other mouse strains called BL/6 and SJL. Therefore a preference for non-ulcerogenic adjuvants will be made for the immunisation of non-BALB/C strain mice. If Freunds Complete Adjuvant is used for weak antigens such as proteins, it will only be used once, only via the subcutaneous route and the maximum administered volume per animal will be 0.1 ml for Protocol 1 and 0.05 ml for Protocol 2. The intravenous route will be reserved exclusively for genetic (DNA) immunisations and no adjuvants will be used. Volumes of antigen preparation for administration will not exceed 0.2 ml and adhere to published and AWERB guidelines. The maximum allowable blood sample to be withdrawn will be 10% of the total blood volume complying with published and AWERB guidelines.
The PPL holder will attempt to refine procedures throughout the lifetime of the licence by staying abreast of published literature and latest developments, online resources belonging to organisations such as NC3Rs, Institute of Animal Technology (IAT) and The Laboratory Animal Science Association (LASA), consulting with other experts and attending relevant scientific meetings such as those organised by the AWERB.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I will adhere to the advice within the publication Turner. P.V. et al., 2011, JAALAS, 50, 600-613.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Attending 3R themed lectures organised by my AWERB.
Regular review of the AWERB's Named Information Officer's (NIO) local SharePoint database. Consulting with other groups and scientists involved in this type of work.
Consulting with Home Office inspectors.
Regular review of the NC3Rs, Institute of Animal Technology (IAT) and The Laboratory Animal Science Association (LASA) websites.

92. Long term effects of in vitro fertilisation on the maternal and fetal cardiovascular system

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

in vitro fertilisation, maternal and fetal health, Developmental hypoxia, Preeclampsia, culture conditions

Animal types	Life stages
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult
Zebra fish (Danio rerio)	Embryo and egg, Neonate, Juvenile, Adult
Killifish	Embryo and egg, Neonate, Juvenile, Adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to assess the long term effect of in vitro fertilisation on maternal and fetal cardiovascular health. We are interested in this topic from a clinical perspective, because we are trying to develop maternal therapeutics that will protect babies and mothers from developing cardiovascular disease in later life.
Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The use of in vitro fertilisation (IVF) has increased exponentially since it was first developed over 40 years ago. While the technique is generally accepted as safe, there are some concerns about the long term effects of IVF on maternal and fetal health. For example, some studies have suggested women that undergo IVF are 27% more likely to develop hypertension or have a stroke in later life.
Furthermore, there have been reports of cardiovascular abnormalities in children that came from IVF pregnancies. It is not known whether these children go on to develop cardiovascular disease later in life because the oldest person to have come from an IVF pregnancy is only 46. Therefore, it is important to determine the long-term effects of IVF on the cardiovascular system of mothers and babies we can develop treatments to protect them from heart disease.

What outputs do you think you will see at the end of this project?

Data streams will be generated in the form of in vitro measurements of cardiovascular function which will be used in my laboratory, shared with academic collaborators and published in peer-reviewed journals. The outputs will be used to identify drug targets for therapeutic intervention. In the long-term, we envisage sharing the data with industry partners and the pharmaceutical industry to develop therapies for protecting humans from cardiac disease. All of the findings will be published in peer- reviewed leading scientific and clinical journals as appropriate to ensure wide dissemination of the research findings.

Who or what will benefit from these outputs, and how?

This project has several expected benefits:

- Short term: Basic scientists and clinicians will benefit from understanding disease mechanisms and the long-term effects of IVF on maternal and fetal health

- Medium term: Clinicians will benefit from the identification of cellular targets for drug intervention in IVF pregnancies.

- Long term: People will benefit from the development of therapeutic interventions that protect mothers and babies from developing heart disease in later life

How will you look to maximise the outputs of this work?

1. Dissemination of new knowledge at national and international conferences (posters, oral presentations, workshops)
2. Participation in public engagement activities, such as science fairs
3. Dissemination of multimedia material through digital media such as laboratory websites and social networks
4. Publication in high-impact open-source scientific journals
5. Publication of datasets in open-source repositories
6. Engagement with patient associations (societies, charities, hospitals)
7. Engagement with industry partners and policy makers to refine and regulate IVF culture media
8. Engagement with media to raise awareness of IVF research

Species and numbers of animals expected to be used

- Mice: 1400
- Zebra fish (Danio rerio): 12900   Other fish:
- Killifish : 2400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using mice as surrogate animal models for understanding the effects of IVF on the human cardiovascular system. Mice are an excellent surrogate model for studying humans because of their short lifespan, fast generation time and low husbandry costs.
In addition to mice, we are using zebrafish and killifish to identify optimal culture conditions for IVF and study the transgenerational effects of IVF, respectively. This is because these models have transparent embryos (zebrafish and killifish) and extremely fast generation times (killifish), which allow us to monitor embryonic cardiovascular function in vivo, and across generations. While these models are appropriate for this purpose, they can not be used for all of our experimental aims because their cardiac physiology is very different to mammals, and many of the ion channels we are interested are not well-conserved between mammals and fish.

Typically, what will be done to an animal used in your project?

Mice

Typically, in vitro fertilisation will be performed with wild type or mutant mice. The culture conditions of the embryos will be changed to mimic conditions normally found in different IVF clinics. The embryos will be transferred into recipients, and the pregnant mice will be single-housed in standard cages. In some cases, pregnant mice may be given therapeutics to protect the unborn pups, and this will be done via the mothers drinking water. Once pups have been born, we take regular measurements of body weight and closely monitor their health. Mice will have minor procedures including ear biopsy, hair sampling and mouth swabbing. We may also take intravenous blood samples, or inject substances using standard routes (intravenous, subcutaneous, intraperitoneal). Blood pressure and cardiac function may be monitored in the mothers (during and after pregnancy) and the offspring.
Some mothers and offspring may be surgically instrumented with telemetry systems to measure ECG's and induce arrhythmias. Finally, the mice will be killed by a humane method, and tissues will be frozen for further analysis.

Fish

Wild type or genetically modified fish embryos will be exposed to various culture conditions during embryonic and larval development. This will include changes in glucose, oxygen concentration and amino acids. Some of these embryos or larvae will be terminally anaesthetised for a cardiovascular assessment. Others will be returned to normal conditions, and maintained until 18 months of age (zebrafish) or 45% of natural life span (killifish, life span is species-dependent). During this time, fish may have minor procedures including biopsy of caudal fin, swab of surface mucus, fin clipping of larva, and micro-

abrasion of embryos. They may also be subjected to swimming tests where the fish where fish will voluntarily swimming against a current, and a hypoxia challenge where fish will be subjected to low oxygen levels. In both cases, the fish will fully recover from these procedures. Some fish may also be terminally anaesthetised for a cardiovascular assessment. Finally, all fish will be killed by a humane method, and tissues will be frozen for further analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice

We do not expect any adverse effects from the embryonic exposures on fetal health, but the mothers may experience high blood pressure (preeclampsia). After birth, the offspring may be smaller (intrauterine growth restriction). After weaning, the mothers and offspring may have mild cardiovascular abnormalities, such as an increase in systemic blood pressure, but usually the phenotype is not conspicuous unless they are placed under physiological stress. Blood pressure measurements are non-invasive and have no adverse effects beyond handling, and echocardiography

is done under anaesthesia, with only minor discomfort during anaesthetic administration. Animals may experience brief, slight discomfort with blood sampling and injection of substances, but no lasting harm. The telemetry surgery involves implantation of the probes into the body cavity which causes very little suffering, and triggering of arrhythmias do not cause any noticeable distress or pain.

Fish

We do not expect any adverse effects from the embryonic exposures. The only post-hatch procedure that may cause moderate suffering is the hypoxia challenge test, which has moderate adverse effects

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice = 25% moderate, 75% mild
Fish = 25% moderate, 75% mild

What will happen to animals used in this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animal models are necessary to investigate the long-term effects of IVF because we will be doing measurements of cardiovascular function across the life-course. It is not possible to do these experiments in humans because their lifespan is too long, and it is not ethical to alter the human fetal environment for experimental purposes. We can not use in vitro preparations because we are studying cardiovascular function at multiple life stages (fetal, neonatal, juvenile and adult) which can not be recapitulated in cell lines or organoids. The complexity of the vertebrate cardiovascular system and the longitudinal nature of our study also precludes the use of computer simulations, which are not currently capable of modelling cardiovascular development across the life course. However, we will be assessing some changes at the pre-protected stages as a partial replacement.

Which non-animal alternatives did you consider for use in this project?

Human volunteers or epidemiological data

- In vitro preparations, including cell culture, organs on a chip and organoids
- Computer modelling and simulations
- Non-protected species such as fruit flies or nematodes

Why were they not suitable?

It is not possible to study humans for this set of experiments because we cannot control the developmental environment, and human tissue is; i) of limited availability, ii) rarely not already diseased and iii) nearly always subject to pharmacological interventions. We can not use epidemiological datasets because current repositories lack details on fetal and maternal conditions during pregnancy, and do not extend into late adulthood. Furthermore, these measurements are purely correlative, and do not prove causation. We can not use in vitro preparations because we are studying organ development across the life-course, and it can not be suitably modelled using computer simulations.
Lastly, we cannot use non-protected animal alternatives because we wish our findings to be clinically relevant to human diseases of the heart. However, we are using less sentient species (fish) to achieve some of our experimental objectives.

Exceptions

There are some questions within our study that could be answered using cell lines. For example, if we found that a particular protein had been reduced in the hearts of offspring from IVF pregnancies, we could genetically manipulate heart cell lines (e.g. knockout experiments) to; 1) replicate the condition and confirm causation, and/or 2) test the suitability of therapeutics to restore normal cell function

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Experimental design has been discussed with, and approved by, our statistical advisor. We calculated the minimum number of animals that we would need to produce statistically significant results. To do this, we analysed data from our laboratory and published articles to get an idea of how many animals are usually needed to produce a significant effect. Using this method, we estimate a sample size of 10 (mice) and 15 (fish) is sufficient for most of our measurements. This estimate will be updated and recalculated throughout the project as we generate new data. For longitudinal experiments, we've used our annual return of procedures data to estimate the number of animals that we will need to use for breeding.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have taken measures to reduce the total number of rodents we use by performing intraperitoneal heparin injections prior to humane killing to prevent blood clots forming in the in vitro heart preparations. This procedure improves the success rate of our preparations by 50%, which reduces the total number of animals used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Efficient breeding - our laboratory has been breeding mice and fish for over 10 years, and the animal facility has extensive experience in the mice and fish models we will be using. We have developed effective breeding protocols which usually result in an 80% success rate of pregnancy (mice) or fertilisation (fish).
Pilot studies - we use pilot studies to confirm aspects of our study design, such as drug dosage
Sharing of tissue - we sample multiple organs from each animal after humane killing to answer parallel questions from the same animal

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We are using IVF culture conditions that are currently being used in human IVF clinics and mouse genomic facilities. They are not known to cause any lasting pain, suffering or distress. We have also chosen to use mostly non-invasive cardiovascular measurements to assess cardiovascular structure and function. Having thoroughly reviewed the literature, we are unaware of any other methods that could be used which would cause less suffering to the animal models we are using.

Why can’t you use animals that are less sentient?

Many of our experiments are undertaken at immature life stages and ended before the animals are officially protected. However, because we are interested in the long-term effects of IVF, we must also investigate later life-stages, including fetal, neonatal, juvenile and adults. We can not use less sentient animals for most of our experiments because we want the results to be translationally relevant, and lower vertebrates have significantly different cardiac morphology and physiology. Where possible, we will use fish models to achieve our objectives, and we usually use terminal anaesthesia for cardiovascular assessments. However, in some cases, it is extremely important to obtain in vivo measurements without anaesthesia, because anaesthetic agents strongly affect the cardiovascular system and mask the responses we are trying to measure.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice

Mice cages will include environmental enrichment (material to gnaw, refuges and nesting material), and solid floors with appropriate levels of substrate and appropriate lighting levels. It is not possible to house pregnant mice in groups as we need to monitor food and water intake in order to characterise the phenotype of the rodent. However, once pups have been weaned, mice will be housed in stable, compatible groups, taking into account sex, age, reproductive condition, familiarity, and prior group housing. We will regularly monitor the animals and use body condition scoring (BCS) to evaluate overall condition. Animals will be given analgesia when needed. Control rodents will be used to assess the normal body weight and BCS changes during pregnancy; this will be a separate group of pregnant rodents that will not be subjected to any procedure throughout the pregnancy.
Fish

We not expect any welfare costs for fish in these experiments, apart from the hypoxic challenge, which can lead to erratic behaviour as critical oxygen tensions are approached.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We consult the PREPARE guidelines to design our studies (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence). This has allowed us to refine three areas of our study design; formulation of the study, dialogue between scientists and the animal facility, and quality control of the components in the study. We will continue to consult the guide as it becomes updated.
We also adhere to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines when publishing our research in order to maintain the highest standard of study design, statistical analysis and animal reporting.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We regularly consult information portals (e.g. www.nc3rs.org.uk, www.lasa.co.uk), published guidelines (e.g. https://journals.sagepub.com) and academic journals (e.g. Animals, Animal Welfare) to stay informed about advances in the 3R's, and to identify new

techniques and protocols. We hold an account with the NC3R's where we receive regular newsletters, and our team continues to attend and present at NC3R symposiums.

93. Long-term effects of a reduction in fetal oxygen supply on the maternal and fetal cardiovascular system

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Developmental programming, Cardiovascular, Developmental hypoxia, Preeclampsia, Maternal and fetal health

Animal types	Life stages
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult
Rats	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to assess the long term effect of a reduction in fetal oxygen supply on the maternal and fetal cardiovascular system. We are interested in this topic from a clinical perspective to develop maternal therapeutics that protect mothers and babies from developing cardiovascular disease in later life.
Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Women often experience conditions during pregnancy that reduce oxygen supply to the developing embryo and fetus. The lack of oxygen (termed hypoxia) can permanently alter the cardiovascular system of the fetus, and can increase the chances of maternal preeclampsia. Both of these problems can increase the likelihood that the mother and child will develop cardiovascular disease later in life. Therefore, it is important to develop novel therapeutics that can protect the mother and unborn fetus from hypoxia and prevent heart disease in later life.

What outputs do you think you will see at the end of this project?

Data will be generated in the form of measurements of cardiovascular function which will be used in my laboratory, shared with academic collaborators and published in peer-reviewed journals. In the long-term, we envisage sharing the data with industry partners and the pharmaceutical industry to develop therapies for protecting humans from cardiac disease. All of the findings will be published in peer-reviewed leading scientific and clinical journals to ensure wide dissemination of the research findings.

Who or what will benefit from these outputs, and how?

This project has several expected benefits:

- Short term: Basic scientists and clinicians will benefit from understanding the long-term effects of low fetal oxygen supply on disease mechanisms, and maternal and fetal health

- Medium term: Clinicians will benefit from the identification of cellular targets for drug intervention in hypoxic pregnancies.

- Long term: People will benefit from the development of therapeutic interventions that protect mothers and babies from developing heart disease in later life

How will you look to maximise the outputs of this work?

1. Dissemination of new knowledge at national and international conferences (posters, oral presentations, workshops)
2. Participation in public engagement activities, such as science fairs
3. Dissemination of multimedia material through digital media such as laboratory websites and social networks
4. Publication in high-impact open-source scientific journals
5. Publication of datasets in open-source repositories
6. Engagement with patient associations (societies, charities, hospitals)
7. Engagement with industry partners and policy makers to refine and regulate IVF culture media
8. Engagement with media to raise awareness of IVF research

Species and numbers of animals expected to be used

- Mice: 850

- Rats: 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using mice and rats as surrogate animal models for understanding the effects of the developmental hypoxia on the human cardiovascular system. Rodents are an excellent surrogate model for studying humans because of their short lifespan, fast generation time and low husbandry costs. In some experiments, mice are a better choice than rats because their genome can be easily manipulated. On the other hand, rats are larger than mice, and provide larger tissue samples which reduce the amount of animals necessary for experimentation; this is particularly important for our in vitro studies. Lower vertebrate models are not appropriate for these experiments because their cardiac physiology is very different to mammals, and many of the proteins we are interested are not well-conserved between mammals, fish, amphibians and reptiles.

Typically, what will be done to an animal used in your project?

Typically, pregnant rodents will be single-housed in standard cages and moved into an environmental chamber during pregnancy. Oxygen levels in the chamber will be decreased to simulate fetal hypoxia. In some cases, animals may be given therapeutics to protect the unborn pups and mothers from hypoxia, and this will be done via the mothers drinking water. We will then remove the animals and transfer them to normal cages 1 day before they are due to litter. Once pups have been born, we take regular measurements of body weight and closely monitor their health. Some animals will have minor procedures including ear biopsy, hair sampling and mouth swabbing. We may also take intravenous blood samples, or inject substances using standard routes (intravenous, subcutaneous, intraperitoneal). Blood pressure and cardiac function may be monitored in the mothers (during and after pregnancy) and the offspring. Some mothers and offspring may be surgically instrumented with telemetry systems to measure ECG's and and induce cardiac arrhythmias.

What are the expected impacts and/or adverse effects for the animals during your project?

Pregnant rodents can experience moderate adverse effects from hypoxia, such as a transient or sustained reduction in activity or appetite, as well as pre-eclampsia like symptoms (e.g. increased blood pressure). Fetal hypoxia can cause offspring growth restriction, but this usually resolves after weaning. Other than that, there are no overt signs of any morphological or functional abnormalities in the offspring, and no signs of distress. Blood pressure measurements are non-invasive and have no adverse effects beyond handling, and echocardiography is done under anaesthesia, with only minor discomfort during anaesthetic administration. Animals may experience brief, slight discomfort with blood sampling and injection of substances, but no lasting harm. The telemetry surgery can induce moderate adverse effects due to the implantation of the probes into the body

cavity (which can cause post-operative pain), but triggering of arrhythmias do not cause any noticeable distress or pain.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice and rats: 68% mild, 32% moderate

What will happen to animals used in this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animal models are necessary to investigate the long-term effects of fetal hypoxia because we need to make measurements across the life-course. It is not possible to do these experiments in humans because their lifespan is too long, and it is not ethical to alter the human fetal environment for experimental purposes. We can not use in vitro preparations because we are studying cardiovascular function at multiple life stages (fetal, neonatal, juvenile and adult) which can not be recapitulated in cell lines or organoids. The complexity of the vertebrate cardiovascular system and the longitudinal nature of our study also precludes the use of computer simulations, which are not currently capable of modelling cardiovascular development across the life course.

Which non-animal alternatives did you consider for use in this project?

- Human volunteers or epidemiological data.
- In vitro preparations, including cell culture, organs on a chip and organoids
- Computer modelling and simulations
- Non-protected species such as fruit flies or nematodes

Why were they not suitable?

It is not possible to study humans for this set of experiments because we cannot control the developmental environment, and human tissue is; i) of limited availability, ii) rarely not already diseased and iii) nearly always subject to pharmacological interventions. We can not use epidemiological datasets because current repositories lack details on fetal and maternal conditions during pregnancy, and do not extend into late adulthood. We can not use in vitro preparations because we are studying organ development across the life-course, and it can not be suitably modelled using computer simulations. Lastly, we cannot use non-protected animal alternatives because we wish our findings to be clinically

relevant to human diseases of the heart, and the use of other less sentient species, such as ectotherms and nonprotected species, is usually not appropriate for the main study animal as their hearts differ significantly from mammalian hearts.

Exceptions

There are some questions within our study that could be answered using cell lines. For example, if we identify drug targets, we could genetically manipulate cell lines (e.g. knockout experiments) to confirm that our targets are important before moving onto animal studies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Experimental design has been discussed with, and approved by, our statistical advisor. We calculated the minimum number of animals that we would need to produce statistically significant results. To do this, we analysed data from our laboratory and published articles to get an idea of how many animals are usually needed to produce a significant effect. Using this method, we estimate a sample size of 10 is sufficient for most of our measurements. This estimate will be updated and recalculated throughout the project as we generate new data. For longitudinal experiments, we've used our annual return of procedures data to estimate the number of animals that we will need to use for breeding.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have taken measures to reduce the total number of rodents we use by performing intraperitoneal heparin injections prior to humane killing to prevent blood clots forming in the in vitro heart preparations. This procedure improves the success rate of our preparations by 50%, which reduces the total number of animals used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Efficient breeding - our laboratory has been breeding mice and rats for over 10 years. We have developed effective breeding protocols which usually result in an 80% success rate of pregnancy.

Pilot studies - we use pilot studies to confirm aspects of our study design, such as drug dosage

Sharing of tissue - we sample multiple organs from each animal after humane killing to answer parallel questions from the same animal

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

In most cases, the level of hypoxia we will use is very mild (13% oxygen), similar to what you would find in high-altitude cities, such as La Paz (Bolivia). The more moderate levels of hypoxia (10% oxygen) that we use can cause more adverse effects, but these are usually transient for the mother, and do not produce any overt cardiovascular phenotype in the offspring. We have also chosen to use mostly non-invasive cardiovascular measurements to assess cardiovascular structure and function.
Having thoroughly reviewed the literature, we are unaware of any other methods that could be used which would cause less suffering to the animal models we are using.

Why can’t you use animals that are less sentient?

Many of our experiments are undertaken at immature life stages and ended before the animals are officially protected. However, because we are interested in the long-term effects of developmental hypoxia, we must also investigate later life-stages, including fetal, neonatal, juvenile and adults. We can not use less sentient animals because we want the results to be translationally relevant, and lower vertebrates have significantly different cardiac morphology and physiology. Where possible, we always use terminal anaesthesia for cardiovascular assessments. However, it is extremely important to obtain in vivo measurements without anaesthesia, because anaesthetic agents strongly affect the cardiovascular system and mask the responses we are trying to measure.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We use a specially designed hypoxic chamber that provides the animals with the most comfortable environment as possible. Rodent cages will include environmental enrichment (material to gnaw, refuges and nesting material), and solid floors with appropriate levels of substrate and appropriate lighting levels. It is not possible to house pregnant rodents in groups as we need to monitor food and water intake in order to characterise the phenotype of the rodent. However, once pups have been weaned, rodent will be housed in stable, compatible groups, taking into account sex, age, reproductive condition, familiarity, and prior group housing. We will regularly monitor the animals and use body condition scoring (BCS) to evaluate overall condition. The animals will be given analgesia when needed. Control rodents will be used to assess the normal body weight and BCS changes during pregnancy; this will be a separate group of pregnant rodents that will not be subjected to any procedure throughout the pregnancy.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We consult the PREPARE guidelines to design our studies (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence). This has allowed us to refine three areas of our study design; formulation of the study, dialogue between scientists and the animal facility, and quality control of the components in the study. We will continue to consult the guide as it becomes updated.
We also adhere to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines when publishing our research in order to maintain the highest standard of study design, statistical analysis and animal reporting.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We regularly consult information portals (e.g. www.nc3rs.org.uk, www.lasa.co.uk), published guidelines (e.g. https://journals.sagepub.com) and academic journals (e.g. Animals, Animal Welfare) to stay informed about advances in the 3R's, and to identify new techniques and protocols. We hold an account with the NC3R's where we receive regular newsletters, and our team continues to attend and present at NC3R symposiums.

94. Morphogenesis of the early mammalian embryo

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

developmental biology, morphogenesis, mechanical forces, organogenesis, tissue development

Animal types	Life stages
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult
Rats	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand how mechanical forces shape complex three-dimensional structures such as tissues and organs out of simple cell populations using the early mouse and rat embryo.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Birth defects, or congenital abnormalities, are one of the leading causes of infant mortality across the world. While some of these abnormalities can identified early in pregnancy, we have only limited ability to repair such defects in the womb. This is largely because we have only a poor understanding of how tissues and organs develop in the embryo. Additionally, hundreds of thousands of people who are in need of new organs wait on long transplant lists, relying entirely on donors, and face the possibility of having to take

immune-suppressant drugs for the rest of their lives. For both sets of patients, infant and adult, their options would be greatly expanded if we were able to grow new tissues and organs outside of the body. However, while we have made significant advances in understanding how genes are regulated throughout development, and using stem cells and organoids can differentiate specific cell types and populations, we are not yet able to build more elaborate structures such as organs or even multi-layered tissues.
The work we propose will allow us to determine how the early embryo grows and develops from simple, homogeneous starting materials; how cells are organized to form tissues, how tissues coordinate to create layers and shapes, and what mechanical forces are required to fold these tissues into a functioning, moving organ such as a beating heart. Only with this knowledge can we begin to replicate these processes ourselves, using cell culture or organoids to generate tissues and complex shapes, to know when and where to manipulate and what to add and what to subtract. The ability to one day repair damaged organs, treat developmental defects in utero, and even grow whole, functional organs outside of the body will depend on our understanding of how the embryo builds such complex structures.

What outputs do you think you will see at the end of this project?

The successful conclusion of this project will result in at least several publications, collaborations, and the generation of a number of datasets and models that will be shared freely with the scientific community. Information gained from experiments will be used as a platform for further, more advanced studies that will continue to build from the previous goals. Our insights and analysis from studying foregut formation and early organogenesis will reveal mechanisms that are likely re-used throughout the development of a wide range of tissues and organ systems.

Very little is known about the cellular mechanisms surrounding early mammalian development during the stage in which the first tissues and organs are formed. This is largely due to our inability to visualize and study these processes live, which we have only now been able to overcome with advances in new imaging technologies. This project will result in a vast amount of imaging data that covers the full scope of mouse and rat embryo development over the crucial organogenesis period. While we focus primarily on the morphogenesis of the foregut and the anterior pattering of the embryo, the data we collect also contains a wealth of information for many other fields of research. By making these datasets available, others will be able to address their own biological questions and formulate new hypothesis without having to generate new datasets themselves. Already our timelapse movies have been re-used in several publications and studies to address a variety of questions, from biological to computational, demonstrating the value of these rich datasets. Our ability to make quantitative measurements on morphogenetic processes will help inform other labs working in synthetic or in silico systems as to what parameters or physical requirements are needed to form different shapes and structures. This will then hopefully translate into being able to generate such complex structures in a dish, with the knowledge gained from our investigations into the mammalian embryo.

Who or what will benefit from these outputs, and how?

In the short-term, datasets that are generated may be shared with collaborators or public databases to be used freely by the scientific community. At the completion of this project, all datasets, computational tools, technical drawings and specifications, computational models and tools used to generate publications or demonstrate advances will be shared

openly with the scientific community. The knowledge gained from this project will enable researchers in other fields to employ new techniques to make more complex structures and further advance the development of their systems. Additionally, advances in imaging and embryo culture resulting from these studies will allow researchers to study later and more complex stages of mammalian development.

How will you look to maximise the outputs of this work?

We actively seek collaboration with other researchers who may have complimentary knowledge or may directly benefit from our studies, as well as ensuring all results and data are made freely and publicly available. Results are shared both in publications and at conferences and meetings that are widely attended by the scientific community. Public engagement activities will help dissemination of the knowledge to the general public.

Species and numbers of animals expected to be used

- Mice: 12000
- Rats: 800

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice and rats have been an excellent model system to study mammalian development for decades; they are prolific breeders, a close analog for human development, and there are many genetic mutants and fluorescent reporter lines available. For our proposals, there are no other animal models that could serve as a viable replacement or give us a closer understanding of human development (save for primate species). We primarily use adult mice or rats to produce embryos that are used in experiments, and embryos before the age of viability in order to study how organs and tissues are formed. While mice have long been used in the field of developmental biology field for the ease in which embryos can be obtained and animals can be genetically modified, rats share many of the same benefits as mice and potentially an even greater similarity to human development. The formation of the placenta in rats, for example, is much closer to human development than it is to mouse. Rat embryos are also more accessible in some areas to live imaging than mice, making it easier to study developmental events such as the formation of complex tissues and organs.

Typically, what will be done to an animal used in your project?

Adult mice and rats will be used to breed to maintain GA lines, or produce embryos either through natural breeding, super-ovulation which involves the injection of hormones at two timed intervals, or through embryo-transfer experiments where female mice or rats are anesthetized and one or two small incisions are made in their back order to place embryos inside the uterus or oviduct. Females may also be used to produce embryos through NSET (non-surgical embryo transfer) or AI (artificial insemination) methods. The majority of animals used will experience only no amount or only very mild pain or discomfort.

Embryo transfer and super-ovulation protocols are only occasionally required, and the majority of experiments will rely on natural breeding of genetically altered mice to produce embryos, and before recovering those embryos the female is first humanely euthanized and experiences no pain or discomfort. Timed mating of rats will include checking for oestrus cycle prior to mating, in order to reduce the number of animals needed for each experiment. Additionally, the pregnancy of female rats can be verified using ultrasound, again to reduce the number of animals used, and some animals may be lightly anesthetized if needed to minimize discomfort.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of the animals will be genetically altered and not expected to experience any harms. A few lines carry genes which may cause a small number of mice to present with clinical signs (such as distended abdomen), these mice are removed from the colonies and culled. Only mild, temporary pain or discomfort is expected for the majority of animals on this project. Animals carrying the LifeAct-RFP mutation have developed an unexpected harmful phenotype, and will be culled as soon as they display symptoms. Newly generated C-YAP mice appear to have a higher incidence of cataracts, but this is not anticipated to cause pain or distress to the animal. The highest degree of pain is experienced for animals undergoing vasectomy or embryo transfer procedures.
These effects are temporary, lasting no more than a day or two and can be managed with pain medication and animals will be monitored like a human patient under a doctor's supervision, and are not expected to have any long term physiological or behavioural effects. Complications from these surgical procedures are very rare in our experienced animal facility.

Vasectomy and embryo transfer procedures are expected to produce an uncomfortable degree of pain and discomfort, which is only temporary and will be managed with pain medication. Only a small number mice will be used for these procedures, and most will only experience either no or only a very mild amount of pain or discomfort. All other mice used in this project involve the breeding of males and females, and with the exception of the LifeACT-RFP line, are not expected to experience any significant level of stress. LifeACT-RFP mice developed an unexpected and unexplained phenotype upon rederivation into our facility, and while the majority of these animals (~75%) are discovered and culled while their symptoms are still mild, some (~25%) present immediately with symptoms of a moderate severity. Symptoms include abdominal bloating, inflammation, and over-all poor body condition. This phenotype is of unknown origin and has not been previously encountered in the line so is unexpected.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Approximately:
Mice:
80% sub threshold, 5% moderate and 15% mild
Rats:
80% sub threshold, 5% moderate and 15% mild

What will happen to animals used in this project?

- Used in other projects
- Kept alive at a licensed establishment for non-regulated purposes or possible reuse

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our understanding of how an organism develops from a single-cell of limitless potential, to all the complex cells, tissues, and organs that comprise an adult animal relies on our ability to observe and interrogate this process in nature. Mouse and rat embryos are a close analogue to human development, sharing many of the same genes and processes that shape organs and tissues. Birth defects seen in humans are also found in rodents, allowing us to study these processes in the hopes of one day being able to correct them in the womb.

Which non-animal alternatives did you consider for use in this project?

Because we understand so little about mammalian development, there are no synthetic or artificial alternatives that can replace actual embryonic development. Research stem-cell based models that aim to recapitulate embryonic development such as gastruloids and organoids present the closest non- animal analogue, however this field is still in a very young stage, and indeed would stand to benefit from the results of this project. Gastruloids, organoids, and newly described synthetic embryos do not fully recapitulate mammalian development, nor do so normally, and additionally require animal-derived products in sometimes large quantities to support their culture.

Why were they not suitable?

The use of non-animal, cell-culture based 3D systems can make approximations of some cell types or structures, but they do not form normal embryos or even functional organs, nor can they be used to validate "normal" development as we do not yet even understand what "normal" development is.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This number was estimated from similar experiments performed over the past five years, taking into account the rate of failure, the frequency of false pregnancies, and the

estimated number of embryos and observations that are needed in order to make statistically sound conclusions.

Due to the number of different fluorescent reporter lines that are required to visualize various developmental processes, we have over 30 different mouse strains that are housed within our facility, with cage limits kept as small as possible to maintain the lines. Females are used most frequently to produce embryos for experiments, with typically 5-15 females being used each month. Around 40% of these will not be pregnant. Some litters do not produce enough females for our current need, and subsequent litters are planned as a result. Because females are most effective at breeding while they are young, a constant supply breeding-age females is required.

We have only one fluorescent rat strain at the moment, and use on average around 5 females a month. Currently the chance of pregnancy is less than 50%, however use of methods such as pro-oestrous cycle screening and ultrasounding females is helping to improve this number.

The number of experiments needed to ensure confidence in observations and measurements is also guided by accepted numbers commonly used in literature. More conventional power calculations are not suitable in this case as much of the work is based on discovery and observation or measurements in an inherently stochastic system. It is not appropriate to employ standard deviation as a measure of confidence in a system where that standard deviation may instead represent the normal, natural variation.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

While it is impossible to control whether or not animals become pregnant nor the number of embryos they produce, we are able to estimate based on expected rates of failure generated from years of previous data for similar experiments, combined with the NC3R's Experimental Design Assistant where appropriate, the number of animals that would be required to confirm observations and measurements in a statistically significant way.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Ensuring a controlled breeding environment will help to reduce the number of false pregnancies. In certain cases, ultrasound can be used to verify pregnancy, reducing the number of females that are sacrificed unnecessarily for each experiment. We also try to use non-surgical methods to generate founders and re-derive lines using non-surgical embryo transfer (NSET) or sperm transfer (Artificial Insimination), which reduces the over-all number of female mice and rats needed. Additionally, females used for breeding and super-ovulations can be re-used in the same or other protocols. Continuous data analysis on results as they are obtained will allow us to continually refine the number of animals that are needed in order to achieve a statistically significant result.
In addition, Wild-type and GM rats generated through breeding can be used to collect serum needed for embryo culture, reducing the number of animals that are needed to be ordered in through a supplier. Female rats undergo oestrous cycle monitoring, which ensures that only females receptive to mating are used in timed-matings, reducing the number of females that are required to produce embryos.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use mice and rats to produce early-stage embryos in order to study mammalian development. The vast majority of our protocols are designed to only cause minor, temporary, or no pain or discomfort to the animal, and no more than is expected to be caused by natural breeding behaviours. Where possible, we imply methods such as ultrasound to check for pregnancy and is non-invasive, or pro-oestrous checking in the case of rats in order to only use animals which are receptive to mating. Super-ovulation of females can aid in the number of embryos obtained, however the vast majority of our matings are done via natural matings.

In order to generate new transgenic lines or re-implant embryos non-surgical methods such as non- surgical embryo transfer (NSET) or artificial insemination (AI) can be used. In the case of more invasive procedures such as surgeries to implant embryos, or vasectomize males, these are one-time experiences, which will be closely monitored by trained animal staff and technicians in order to minimize any pain or discomfort experienced by the animal.

Why can’t you use animals that are less sentient?

Mice and rats are a close, non-primate analogue to human development, and an excellent model organism that has been well-studied for decades. Our understanding of mammalian development is so limited that there is no viable alternative that would achieve the same amount or quality of results.
The embryos that are used in our program are additionally harvested before legal protections are required, and are not considered sentient.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be closely monitored whether it is for surgical procedures or during the course of normal breeding and husbandry. Changes in breeding behaviour for example can indicate a change in environmental conditions that causes stress for the animals, which can be identified and corrected. Additionally, we employ non-surgical methods wherever possible, as in the case of artificial insemination by transferring sperm through NSET. Local best practice guidance will be used unless stated. This guidance is reviewed annually by the AWERB and experienced NACWOs and veterinarians and reflects best practice from across the literature.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Surgery will be carried out according to the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017). In addition references such as A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes by Diehl et al. 2001, resources from NC3Rs (National Center for the Replacement, Refinement, and Reduction of Animals in Research, https://nc3rs.org.uk/3rs-resources), P. Flecknell (2015) Laboratory Animal Anaesthesia (Fourth Edition),
S.L. Hoogstraten-Miller & P.A. Brown (2008), J. Bruce et al (2001), and other accepted literature as guided by the NVS. Additionally ARRIVE and PREPARE guidelines are used.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The rodent development field is constantly evolving, and new breakthroughs are announced regularly in literature. Additionally, animal care staff are highly-trained and stay abreast of advancements in

animal husbandry, bringing new refined techniques and procedures to scientists and assisting us with their implementation. Additionally, the NIO, NACWO, NVS and others share in the 3Rs, and participate in NC3Rs.

95. Mechanisms of Progressive IgA nephropathy

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

IgA nephropathy, kidney disease

Animal types	Life stages
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The goal of this project is to study the reasons why certain proteins build up in the kidney's filters, leading to damage. This condition, known as IgA nephropathy, is a type of kidney disease. By understanding these reasons, we aim to find specific areas we can treat and test new treatments to slow down or stop the disease from getting worse.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

IgA nephropathy (IgAN) is a common type of kidney disease that occurs worldwide. About 30% of people with this disease may face serious kidney failure, requiring intense treatments like dialysis (a machine that cleans your blood) or a kidney transplant, which can lower their quality of life. Currently, there's no cure for IgAN. This disease develops

when a protein called IgA builds up in the tiny filters inside the kidneys. However, not everyone with this condition experiences worsening symptoms. The greatest risk arises when the kidneys start to scar.

This project builds on our previous work, where we studied IgA nephropathy using genetically modified mice. These mice had a change in a protein that is part of our immune system, leading to increased activity of this system, which can cause inflammation and damage to healthy tissues

In our previous research, we found that certain lab mice developed more severe kidney issues, showing increased levels of a protein and immune response in their blood. In this project, we aim to examine closely what happens after this protein accumulates in the kidneys. We suspect that a part of the immune system, known as the complement system, might significantly worsen the damage. There could be other contributing factors, such as the protein itself, that may also cause kidney scarring by affecting certain cells in the kidney.

Our plan is to study mice that show different levels of activity in this immune system to better understand how kidney damage occurs in our model of the disease. We will also study mice that lack certain proteins in this immune system to see if blocking these parts can protect against inflammation and kidney damage.

If we discover that this immune system plays a crucial role in the disease, we will explore new medications that can inhibit this system, aiming to develop treatments for people with this kidney disease. Additionally, we'll use normal mice (without genetic alterations) to investigate what happens if we prevent the production of this problematic protein.

What outputs do you think you will see at the end of this project?

We're exploring how a specific protein, IgA, contributes to kidney scarring—a serious problem for people with a condition known as IgA nephropathy. Our primary goal is to find ways to slow down or prevent this scarring to help people avoid complete kidney failure.

We are also investigating whether certain factors, like the presence of a specific receptor on kidney cells that the IgA protein attaches to, might make some individuals more prone to severe kidney disease. Understanding this could enable doctors to predict the course of the disease early on and tailor treatments to better suit each patient.

Our research aims to provide new insights into how IgA interacts with other parts of the kidney. We are particularly interested in its relationship with the immune system, cells in the kidney that help produce urine, and the processes that lead to inflammation and scarring. This knowledge could lead to better strategies for managing and treating this disease.

We expect to learn new details about how the protein IgA forms in the body. This understanding will not only deepen our knowledge of the disease but might also lead to innovative treatment approaches. We hope to identify specific areas within the disease process where new treatments could be effective.

Our research will clarify how kidney function changes when IgA accumulates and whether we can reverse or improve these changes to help patients. We're particularly focused on how the immune system contributes to kidney damage in these cases.

Studies are already investigating drugs that block parts of the immune system in other kidney conditions. Our findings might indicate that these drugs could also be effective for treating IgA nephropathy.

Ultimately, the insights gained from our research could rapidly lead to clinical trials for new drugs aimed specifically at IgA nephropathy. Our research will also help us learn how different kidney cells behave and interact when IgA accumulates, leading to kidney damage. This knowledge will expand what we know about the disease, helping us and others understand it better.

We plan to share what we discover with other scientists by publishing in scientific journals and presenting at conferences.

Additionally, the model we are developing will be valuable for other researchers and companies who are looking to test new treatments for IgA nephropathy. We are already collaborating with some companies that are interested in using our model for their testing.

Who or what will benefit from these outputs, and how?

1. Patients: Benefits to patients could include development of new treatments, tools to diagnose and predict the course of the treatment and improved disease management.

2. Researchers and Scientists in Kidney Health: The findings from our study will significantly expand our understanding of kidney diseases, particularly IgA nephropathy. This will be valuable for scientists researching kidney health and how the body fights these diseases. It could lead to new treatments for IgA nephropathy and possibly other kidney conditions.

3. Doctors and Healthcare Workers: This research is directly relevant to medical professionals who diagnose and treat IgA nephropathy. By identifying new aspects of how this disease operates, they could develop better diagnostic tests, monitor the disease more accurately, and tailor treatments more closely to individual patients' needs. This could improve the variety and effectiveness of treatment options available, leading to better patient outcomes.

4. Pharmaceutical Industry and Drug Developers: The insights gained could be crucial for companies developing medications. Identifying new potential treatment targets may lead to the creation of specific drugs for IgA nephropathy, encouraging more investment in research and development of new therapies.

5. Broader Impacts: While our research focuses on IgA nephropathy, the knowledge gained could also enhance our understanding of other kidney diseases and immune-related kidney damage.

Our expectations for this research are high. We believe that within 5 years, we could identify new targets for drug development. Then, in the next 10 years, these findings could advance into clinical trials, where they would be tested in patients.

How will you look to maximise the outputs of this work?

To make sure we get the most benefit from this project, we plan to:

Collaborate Broadly: We'll work closely with other researchers, companies, and organizations. Collaborating allows us to combine expertise, share knowledge, and accelerate progress.

Publish and Present Our Findings: We are committed to communicating our discoveries to the scientific community. We'll do this by publishing detailed articles in peer-reviewed scientific journals and presenting our research at scientific conferences and meetings.
Share Our Challenges: It's also vital to discuss what didn't work. If our experiments don't yield the expected results, we'll share these experiences too. Documenting and publishing our challenges can provide valuable lessons for others in the field, helping them avoid similar pitfalls and refine their approaches.

Species and numbers of animals expected to be used

- Mice: We expect to use up to 500 experimental mice over the course of this license (5 years) for which we would need to breed around 1920.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We use mice in our research for several reasons. First, mouse kidneys function similarly to human kidneys, which makes them a good model for studying kidney diseases. Also, mice have much shorter lifespans than humans, allowing us to observe how kidney disease develops over a quicker timeframe.

The specific type of mice we've chosen is already widely used in research centers globally. These mice are known for developing a predictable type of kidney disease that causes only mild and temporary symptoms, without progressing to severe kidney damage.
We focus on adult mice, aged between 8 and 12 weeks, because by this age their kidneys are fully developed. This maturity provides more reliable and relevant data compared to younger mice. Studying adult mice also gives us insights that are more applicable to adult humans, especially since the kidney disease we are examining typically begins in early adulthood. By using these mice, we can also explore specific genetic changes that could enlighten us further about how kidney scarring happens in this disease.

Typically, what will be done to an animal used in your project?

Under Protocol 1:

We will breed two types of mice: conventional (wild type) mice and genetically altered (GA) mice with specific changes in their genes. These genetic changes do not harm the mice. To check their genes, we will take a small sample from their ears.

Under Protocol 2:

We will give adult mice a substance to trigger an immune response, usually by adding it to their drinking water for several weeks. Then, we will give them daily injections of the same substance (or another one known to cause an immune response) for several days.

Once the mice have developed an immune response, we will give them treatments (or placebo treatments) through injections. We will also collect their urine during the study to monitor their health. This will be done before we start the treatments and after they are completed. The mice will be placed in special cages that allow us to collect their urine easily for up to 24 hours.

The entire study will last about 9 weeks. At the end of the study, we will take blood samples from the mice by carefully puncturing the heart or major blood vessels. The mice will then be humanely euthanized following approved methods whilst they remain under anaesthesia.

What are the expected impacts and/or adverse effects for the animals during your project?

Protocol 1

Based on previous studies, we expect that the animals used in this research will not show any serious adverse effects from breeding. If any adverse effects occur, we will consult our veterinary and animal care staff. For animals with altered immune systems, we will change our care practices to reduce their risk of getting sick.

Protocol 2

The most invasive procedures the animals will undergo include: Intravenous injections (injections into a vein).

Subcutaneous injections (injections under the skin).
Single housing in metabolic cages for up to 24 hours (to collect urine). Intraperitoneal injections (injections into the body cavity).
Blood sampling.

If an animal shows signs of poor health (such as raised fur, hunched posture, abnormal movement, reduced activity, or loss of appetite) that do not improve after seeking advice from our veterinary staff, we will provide supportive treatment for 24 hours. If the condition does not improve within 24 hours, the animal will be humanely euthanized using approved methods. Additionally, any animal that loses 15% of its body weight will be euthanized. To monitor this, animals will be weighed weekly during the study. If any show signs of concern, they will be weighed twice a week or daily if they exhibit other clinical signs. Weight loss will be tracked against their initial weight or compared to age-matched controls.

To make the animals comfortable while in the metabolic cages, we will add red plastic houses for shelter, similar to their normal living conditions, to reduce stress.
Injections: Animals will be closely monitored for any reactions to the injections. If an adverse effect occurs that cannot be quickly and easily fixed, and after consultation with our veterinary staff, the animal will be euthanized using approved methods.

Withdrawal of Blood: Blood samples will be taken from a superficial blood vessel to assess health parameters. Blood sampling will not exceed 10% of total blood volume at once and

15% in a 28-day period. For frequent sampling, the limit will be less than 1% of total blood volume in 24 hours.

Treatments: We are studying drugs that affect the complement system, part of the body's defense system. These drugs could potentially increase the risk of infections or immune system-related diseases. However, these side effects have not been commonly seen because we use the treatments for a short time. We will take precautions to minimize the risk of infection.

Since we don't have a specific list of symptoms to expect from these drugs, we will monitor the treated animals and those given a placebo using a health chart with a scoring system to detect distress. If new information about possible side effects arises, we will adjust our monitoring accordingly.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild 90%, Moderate 10%.

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to use mice in our research because they allow us to study how kidney damage progresses in a living organism. Non-animal methods can't give us the complete picture. The mouse model we use for studying IgA nephropathy is particularly useful because it mimics the disease in humans. In both mice and humans, the disease shows similar signs, such as the buildup of IgA in the kidneys, the gathering of certain immune cells, and the activation of parts of the immune system.

By using this mouse model, we aim to learn more about what causes kidney damage to worsen and how much the immune system's complement pathway contributes to kidney inflammation. Studying diseases in animals like mice is crucial because it helps us understand the disease better and develop new treatments.

Which non-animal alternatives did you consider for use in this project?

Cell Culture Experiments: These involve growing kidney cells in a lab dish to study them outside of a living organism.
Organs on a Chip: This technology uses microchips designed to mimic the structure and function of human organs, including the kidneys.

Why were they not suitable?

In the first part of our project, we will do lab tests on cells to gather information about how IgA (the protein that causes this kidney disease) interacts with key parts of the cells and the important signals inside them. We will use what we learn from these cell tests to help guide our experiments on mice later on.
Although testing cells in the lab is useful, it doesn't fully show us what's happening in real kidneys.

Here's why:

In a real kidney, damaged cells interact with many different proteins at once, and we can't recreate this exact situation in the lab.
In the kidney, cells come into contact with IgA and other harmful substances on just one side of the cell, and we can't copy this perfectly in a dish.
The way kidney cells interact with other types of cells is crucial for why scarring happens, and we can't accurately replicate these interactions in the lab.

"Organs on a chip" technology is also not suitable because it doesn't include the impact of the complement system, which is an important part of our study.
Due to these limitations with cell tests, we've decided it's important to also use mice to study the disease in a living body, which gives us a better understanding of the whole situation.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

In a previous study, we tested if female mice could be used for our kidney disease experiments. We found that female mice did not show the IgA build-up in the kidneys that we wanted to study, but male mice did. Also, reseach shows that human females with IgA Nephropathy have better kidney function than males when they are diagnosed. Therefore, we can only use male mice for this research.

When we breed these mice, only about half of the offspring are male, and of those, only half will have the specific genetic traits (heterozygotes) we need for our experiments. To get enough male mice with the right genetics, we will need to breed up to 1920 mice.

mice

mice

mice Nephropathy

mice Nephropathy

Transgene 1

None Drug 8

Transgene 1
Transgene 1

IgA Nephropathy
IgA Nephropathy

Placebo 8
Drug 8

Experiment 3 Transgene
2

Total 32
None Placebo
None Drug

IgA Nephropathy
IgA Nephropathy

Placebo
Drug
Total 32
Total per year 96

Total over 5 years

480

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We sought advice from our university's Biomedical Services Division and a biostatistician to ensure we use the minimum number of animals needed for reliable results.

To make sure our results are accurate, we designed our experiments to have a 95% confidence level (only a 5% chance that our results are due to random chance) and an 80% power (an 80% chance of detecting a real effect if there is one). The numbers and effect size are based on ongoing human clinical trials and the effects with existing treatments as well as on a previous study in rats (see below for research references). We we determined that we need about 8 mice per group to detect a 40% decrease in a kidney

protein called fibronectin in our genetically modified mice compared to regular mice. This number is similar to the rat study.

For each experiment, we will write a detailed plan that includes:

- Our goals.
- The procedures we will follow.
- The number of mice we will use.
- How we will analyse the data.

We will also specify the statistical tests we will use to determine if our results are significant (not due to chance). We will randomly assign mice to different groups and ensure our study is blinded to reduce bias. This careful planning ensures our experiments are well-designed, meet high-quality research standards, and can be published.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Animals will be supplied by our research facility. We will carefully manage our mouse colony and plan our breeding to ensure we do not produce more mice than we need.
We will calculate the minimum number of mice required to obtain reliable results. After the experiment, we will closely examine the mice's kidney tissue and other samples to gather as much information as possible. This thorough analysis helps us make the most of each experiment and avoid wasting animal lives.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use a well-known strain of mice that scientists all over the world use. This mouse model develops a type of kidney disease that is predictable and causes minimal suffering. These mice do not develop advanced kidney disease. We chose this model to study genetic changes that help us understand kidney scarring better.
We will take several steps to ensure the mice are comfortable:
All procedures will be performed by trained and competent personnel using the latest best practices.

Mice will be housed in groups unless they start fighting and need to be housed separately. They will only be housed alone when placed in metabolic cages.
We will use tunnel handling to reduce anxiety in the mice.

We chose this mouse model because it is the best for studying IgA nephropathy, the kidney disease we are focusing on. It allows us to study mice that have been genetically

modified to lack certain components that interact with IgA in their kidneys, helping us learn more about how IgA affects the kidneys.

Why can't you use animals that are less sentient?

We can't use animals at a younger life stage because IgA nephropathy typically appears in young adults, and using younger animals wouldn't accurately show the disease as it occurs in adults.

Using species that are less sentient wouldn't provide accurate results either. The anatomy and physiology of less sentient animals are quite different from humans, especially regarding the complex interactions between the kidneys and the immune system.
We also can't use animals that are terminally anesthetised (put to sleep permanently) because they can only be used for short experiments. Our protocols need to run for several weeks to study the disease properly.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will keep up to date with new practices and follow the LASA guidelines.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

1. The ARRIVE guidelines: The ARRIVE (Animal Research: Reporting In Vivo Experiments).
2. The National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs) guidelines:

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Attend Meetings: We will regularly attend the Division of Biomedical Sciences (DBS) User Group meetings. These meetings allow researchers to share best practices and get updates on regulations from staff who work with the animals.
Read Scientific Magazines: We will read scientific magazines that feature articles about the 3Rs (Replacement, Reduction, and Refinement). These principles ensure that animals are used in the most ethical way possible in scientific research. For example, we will read "ATLA (Alternatives to Laboratory Animals)" and other publications that include research papers, reviews, and case studies focused on advancements in the 3Rs.
Attend Workshops and Seminars: We will attend workshops and seminars related to animal research and the 3Rs to stay updated on the latest practices and innovations.

Use Online Resources: We will use websites of organizations like NC3Rs, which offer resources, publications, and news related to the 3Rs and also forums that give updates that are more specific to nephrology research such as the Nephrology Networking Group on Linkedin, Labroots – Nephrology Research Group, and the American Society of Nephrology online community section.

Collaborations: We will collaborate with other researchers who study nephrology in animal models.

96. Pathogenesis and treatment of mitochondrial carrier diseases.

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

mitochondria, carrier proteins, citrin deficiency, treatments

Animal types	Life stages
Rats	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult, Aged animal
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult, Aged animal

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to study, and where necessary create, rodent models of mitochondrial diseases, specifically those involving dysfunctional proteins involved in moving small molecules into or out of the mitochondria. The goal is to understand how these diseases develop and to find and test new treatments.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

As the mitochondria are so-called "powerhouses of the cell", problems in these proteins can cause a multitude of different diseases, which are currently poorly understood. One disease of particular interest, citrin deficiency, shows 3 distinct disease presentations at different stages of the patient's life characterised by liver problems in young babies, food aversions and slow growth in children and young adults and liver and neurological symptoms in adults. Due to the fact that these diseases affect multiple organ systems across the body, we need appropriate animal models both to understand further how they develop, and to test potential treatments. The goal would be to help find more appropriate treatments for these conditions, and ultimately a cure.

What outputs do you think you will see at the end of this project?

At the end of this project the direct outputs we foresee are as follows:

1. A rodent model of citrin deficiency. This will either be through confirming the appropriateness of a current commercially available citrin knock-out rodent (rodents lacking the gene encoding the citrin protein) for use in studying the condition, or through generating a new rodent model in house. In particular, this will allow future studies to have a suitable rodent model of the condition on which to work of.

2. A greater understanding of the molecular and cellular basis underlying citrin deficiency. Whilst cell culture models (cells used in the lab that have been generated to lack citrin) have provided some insight into some of the underlying mechanisms in certain cell types, they are unsuitable for determining what is happening across different organ systems and across the different stages of the disease. By having an appropriate animal model for the condition, we will gain greater insight into how the condition develops and progresses.

3. Greater understanding of how treatments currently used for citrin deficiency are working. Whilst some tools have been developed to treat citrin deficiency, there is little understanding as to how they are working in a therapeutic manner, and at what stages of the condition they are working. By applying potential therapies to an appropriate animal model of the condition, we will be able to discover how they are treating the condition, and provide insight to help develop more targeted therapies in the future.

The primary form these outputs will take will be in publications, in peer-reviewed scientific journals. We do not foresee any products being made from the outputs of this project, but aim to gain a greater understanding of both citrin deficiency and potential treatments for the condition, in order to benefit future therapies.

Who or what will benefit from these outputs, and how?

In the short term, the beneficiaries of this research will be other scientists researching citrin deficiency. This is because we will (as publications arise) be providing validation of an appropriate animal model, providing new insight into the underlying mechanisms leading to the condition and how potential treatments work. This research may also be of benefit to other researchers interested in mitochondrial biology and metabolic physiology, as we will uncover further information about the roles mitochondrial carriers play in maintaining healthy functioning mitochondria.

In the longer term, this work will be of benefit to clinicians and clinical researchers involved in treating citrin deficiency. By investigating the molecular and cellular basis to the condition and generating an understanding of how the current treatment options work we will provide an improved foundation from which clinicians can treat the condition. Likewise, this work may benefit researchers in (or interested in) the pharmaceutical industry, targeting citrin deficiency or other mitochondrial carrier (proteins involved in transporting small molecules into an out of the mitochondria, including citrin) diseases. By understanding how the current therapies exert their action, this may pave the way to generate new treatments with greater success in treating the condition and others like it. Ultimately, this will be of benefit to patients and patient families.

How will you look to maximise the outputs of this work?

All new knowledge will be disseminated via open-access publications. We will target journals to ensure that our findings reach the appropriate target audience including both scientists and clinicians working within the field. This will include all findings, regardless of whether they are positive or negative results. We will also present our work at appropriate academic conferences both within the UK and abroad.

Through our continued involvement in the Citrin Foundation, we are part of a global consortium of scientists, researchers and patients all working towards the goal of curing citrin deficiency. As such, we regularly present our work internally within the Foundation, enabling our work to be rapidly disseminated to peers working within the same field. This also enables us to share preliminary results, and gain insight from a network of collaborators to ensure maximal outputs from each experiment.

Species and numbers of animals expected to be used

- Rats: 1060
- Mice: 1620

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using rats and mice from the neonatal stage up to 18 months of age (mice) or 24 months of age (rats).

The disease we are primarily interested in, citrin deficiency, shows a characteristic development of symptoms in patients, that occurs in an age-dependent manner, with newborns and young infants displaying a very different set of symptoms compared with adults and older teenagers. We therefore need to utilise animals at multiple life stages, from neonatal animals to aged rats/mice, to both allow us to understand how the disease develops these distinct stages, and to allow us to test interventional therapies that may be useful in the clinic at the relevant disease stages.

Currently, there is no good model for citrin deficiency. Different commercial models have been developed, but have been incompletely studied to determine whether they are a useful model for the disease. We will comprehensively assess these models, making careful consideration of genetic background and baseline physiological measurements, to allow us to determine the most appropriate model for citrin deficiency. Once we have established the appropriate model, we will enable the total number of animals used to be reduced as experiments will be more reproducible and applicable to the clinical condition.

Typically, what will be done to an animal used in your project?

We will be studying rodent models of citrin deficiency, a mitochondrial carrier disease caused by mutations in a gene encoding a protein called citrin. All rats and mice used will be genetically-altered to remove the citrin gene, or contain a mutated version of the citrin gene seen in human citrin deficiency. This citrin knockout (animal with the gene removed or non-functioning) may be across the whole of the rat/mouse or only in a particular organ. Some mice/rats may have a genetic alteration, which allows the citrin gene to be removed at a specific time during the mouse/rats lifetime (a so called "inducible model"). Additionally, some mice/rats may have other genes removed. These are genes that provide a complementary function to citrin, and therefore can compensate for the removal of citrin in genetically- altered animals. We will utilise littermates that haven't been genetically altered, or only contain one mutated gene copy in our experiments to help us understand the differences between healthy animals and those that develop the disease. Animals may be bred in-house, or may be purchased from a commercial supplier. Animals being purchased will therefore be transported from a supplier (predominantly UK based, but on occasion this may be from abroad) to the animal facility. This can be a stressful procedure for the animals, becoming introduced to a new facility and undergoing transit which can be noisy with less than idealised environmental conditions. Animals bought into the facility will undergo an acclimatisation period (a minimum of 7 days) to allow them to settle into their new environment, prior to any procedure being initiated.
In addition to using genetically-altered animals, the mice/rats may undergo one or more of the following procedures:

- Female animals may receive drugs, delivered under the skin or into the abdomen, to cause them to increase the number of eggs they produce. This will allow us to harvest the eggs, to either store them for future use or generate new genetically-altered models as required. In the latter case, some female animals may undergo embryo transfer, where embryos produced by in vitro fertilisation are re-implanted (non-surgically or surgically under anaesthetic) into a female rat/mouse generating new genetically-altered offspring.

- Animals may be fed a modified diet. This may be both to generate the observable characteristic and clinical signs of the disease (e.g. by using an altered carbohydrate/protein diet) or to test dietary interventional treatments (e.g. altered fat content). Animals may also be exposed to different types of food, or different dietary components (e.g. sugar), including via their drinking water, to determine if they show any preferences for certain food groups, which is a known symptom of citrin deficiency in humans.

- Animals may receive drugs or other compounds known to alter their metabolism. These may be administered as an injection (under the skin, or into a vein, muscle or the abdomen), or orally (by incorporating the compound in their food and water, or

directly to the stomach by a technique called oral gavage) . Again, these may be used to generate or challenge the disease phenotype, or to test drugs/compounds that may have a therapeutic use in treating citrin deficiency.

- Some animals may receive, either via injection (into a vein or the abdomen), oral gavage or inclusion in food/water, molecules called tracers. These allow us to follow the particular metabolic processes, and therefore understand more about what is actually happening in a given tissue.
- Typically, an animal would only receive one of such of these compounds across their lifetime, and would never receive more than five. These compounds are well studied and tolerated well by the rats/mice.
- For short periods of time (usually less than 5 days, but on occasion up to 4 weeks), animals may be single housed. This allows measurements of the individual amounts of oxygen each animal is consuming, their level of activity, accurate food and water intakes as well as urine and faeces production. Additionally, it may be required to single-house animals in order to generate control animals with a matched food intake to another animal in a particular study. In all cases, animals will be rehoused into group housing following the period of single housing, unless this is more stressful for the animals to do so (e.g. causes fighting for particularly aggressive males).
- Some animals may have access to food restricted either completely for a short period of time or partially for a longer length of time. In the first case, this would be to enable us to carry out measurements in fasted animals, similar to how some blood tests require fasting before the blood to be drawn. Whilst in the short-term removing access to food can be distressing to the animal, it causes no lasting harm, and food will always be immediately returned following the experiment (usually withdrawal of blood). In the case of partial food restriction, this will be to ensure we can match food intake between animals in a particular study. Whilst the animals may prefer to have unlimited access to food, the level of food restriction will not be harmful for the animals involved.
- Some animals may be challenged to behavioural and/or exercise tests. This allows us to examine how their fitness level changes during disease progression (exercise) and mental acuity (behavioural testing). Animals would be acclimatised and trained to utilise the test equipment minimising the stress caused. The initial training period may cause some transient stress (as the animals are introduced to a new environment and new equipment), and exercise testing requires the use of a motivational air puff placed behind the animal. However, the most refined methods will be utilised to minimise the stress the animals experience during the testing, and animals will undergo testing as few times as possible across their lifetime, with exercise testing (arguably the most stressful test) likely occurring only once in the vast majority of cases.
- Some animals may be scanned by non-invasive imaging techniques (such as nuclear magnetic resonance or ultrasound) to look at how their bodies or organs are changing during the disease progressing. This may involve brief restraint of the animals, without anaesthetic, in a painless and harmless manner, or alternatively a brief (less than 1 hr) anaesthetic to allow clearer scans to be obtained, without the animals moving around. No animal would be expected to go through more than 6 scans of any type in their life

time, and if an anaesthetic was involved, there would be at least 1 week between each imaging procedure.

- Some animals may have blood periodically withdrawn. This would be from a peripheral vein (such as in the tail or leg), with minimal distress or pain to the animal. Animals are briefly restrained in a tube, whilst the blood sample is drawn. Animals may be warmed prior to the procedure, to encourage the blood vessels to dilate, which makes the finding the veins and withdrawing the blood faster, and thus less stressful for the animals. Multiple blood samples may be required across the lifetime of the animal, but a minimum of a two-week period will be left between samples, with no more 15% total blood volume sampled within a 28-day period.

Whilst a typical animal will experience no more than 5 of these procedures, there may be a cumulative effect of these procedures. The most extreme case would involve the following: a genetically altered animal plus: (a) feeding an altered diet for the entire experiment, (b) single housing the animal for 5- days (twice, with several months in between), (c) anaesthetic administration for non-invasive imaging (three times, with several months in between each administration), (d) an exercise test (with associated training) occurring once and (e) periodic blood withdrawal, approximately once a month.
Despite their clearly being a cumulative effect, we will space out the procedures across the experimental timeline to prevent multiple stressful procedures occurring in quick succession. In this way, we will aim to minimise the cumulative effect and distress associated with the experimental model.

At the end of the experiment, each animal will be killed using the most humane method possible, whilst still allowing collection of blood and tissue samples to not be detrimentally affected for scientific analyses.

What are the expected impacts and/or adverse effects for the animals during your project?

We are aiming to generate and validate a rodent model of citrin deficiency. Rats or mice, if they display a similar disease progression to that of the human condition would be expected to experience three distinct phases of the condition:

1. Newborn animals would be expected to show some signs of liver dysfunction. This may include slight yellowing of the skin (jaundice) and production of a fattier faeces. This phase, if left untreated, would be expected to last until the rats or mice were 3-6 weeks old. Monitoring of the condition would be undertaken, and where necessary, animals would be treated in line with the best practice for treating human citrin deficiency.

2. Animals would then be expected to go through an 'adaptation' period. Here, most animals as they continue to grow would be expected to have very few adverse effects as a result of their genetic mutations. It is possible that some rats and mice may grow slower than if they were not carrying the genetic alteration for citrin deficiency, and they may show some voluntary food aversions, resulting in needing to feed the animals a slightly different diet. A small proportion of these animals may develop some fatigue symptoms, and may have difficulty processing fat, which may result in them becoming lethargic.

3. In older, aged animals, liver dysfunction may again develop. Here, we expect the major change to be in the way the animals are able to handle protein, resulting in the build-

up of a waste product called ammonia in the blood. This may result in the animals experiencing some confusion or disorientation alongside fatigue and lethargy. Urine and faecal output may be altered in these animals.

Some animals will experience just one of these phases, some more than one and some none and all. We expect the minority of animals to experience the final phase, both because it is less frequent to develop in human patients (and we therefore expect it to develop less frequently in any animal model), and because fewer animals will be raised to this age.

In general, the experimental procedures we will be carrying out will either be to (a) test treatments for the condition, and therefore are expected to show less adverse effects or (b) to allow us to track and monitor the changes occurring in the animals as a result of the condition. In this second case, any adverse effects, as outlined below, will be transient and lead to no lasting harm:

- Feeding the animals a modified diet may be required in order to initiate a distinct phase of the disease, and thus may result in an adverse effect outlined above. However, in some cases, modified diets will be used to test potential treatments for the condition, or to ameliorate certain aspects of the disease, and therefore will result in reduced harm to the animals.

- In this project, drugs or compounds known to alter metabolism will typically be utilised to improve the disease condition and therefore would be expected to lessen the adverse effects. Tracers would not be expected to cause any adverse effects. Administering the compound, drug or tracer would not cause any lasting harm from the injection or oral route per se, although the animal may experience a small amount of discomfort during the actual administration.

- Single-housing can be stressful for the animals, but causes no pain or lasting harm. However, times spent in single-housing will be minimised and only carried out when absolutely necessary. Animals will also be kept in close proximity to their original cage mates as we have found this helps to reduce the stress associated with the single-housing in the past. Wherever possible, animals will be rehoused with their original cage mates following the single-housing period. If this is not possible, e.g. for a group of particularly aggressive males which begin to fight upon rehousing, the reintroduction process itself may be distressing, and they may have to remain single-housed, which would also cause some distress. However, all attempts will be made to prevent this occurring and minimising the number of animals that may experience this.

- Behavioural and exercise testing can cause some transient stress to the animals as they are removed from the home cage for testing. However, animals will be acclimatised to all equipment to minimise stress. For some tests (e.g. exercise testing), the training process is associated with some distress, as animals have to explore the equipment which is foreign and potentially uncomfortable. For example, for exercise testing, animals must experience the air puff at the end of a treadmill so they are motivated to run during the testing, and although this is the most refined method od motivating running, it is still an averse stimuli and so it may be distressing to the animals. In all cases, there is no lasting harm from carrying out these tests.

- Non-invasive imaging is harmless and pain-free. Mice and rats that are anaesthetised may feel slightly disorientated and groggy as they recover from the anaesthetic. However, the effects of the anaesthetic are transient and the animals will not suffer lasting harm as a result of the procedure.

- Blood sampling may cause a small amount of discomfort during the actual procedure. However, the animals will not experience any lasting harm as a result of having blood drawn.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice:
Subthreshold: 20%
Mild: 50%
Moderate: 30%
Rats:
Subthreshold: 20%
Mild: 50%
Moderate: 30%

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The conditions associated with mitochondrial carrier diseases are multi-system, and therefore to fully understand these conditions and how to treat them, we need to understand how they progress across organ systems, and which treatments are effective, at which points. In order to do this, we need good animal models for the conditions, which need validation prior to use. Our initial disease of interest, citrin deficiency, is poorly understood and has no good rodent model of the condition. We therefore first need to validate rodent models of citrin deficiency, before we can use it to study the underlying mechanisms of the disease development and progression, and then use these models to develop and test new treatments for the condition.

Which non-animal alternatives did you consider for use in this project?

We have actively been generating cell culture models for citrin deficiency, using established cell lines that we have genetically altered to contain disease carrying mutations. Thus far, we have generated the first and only clonally derived cell line for use in studying citrin deficiency. Using gene editing technology, we have created both

complete and partial knock-outs of the citrin gene in liver cells, which has allowed us to comprehensively characterise the changes occurring in their growth and energy generating pathways as a result of loss of citrin function. We have presented this work at conferences both within the Citrin Foundation (Global Symposium 2023) and internationally (European Bioenergetics Conference 2024). We are also in the process of using newer gene editing techniques to generate cell models of common specific variants of the citrin gene that are seen in patients, so we can understand the differences between complete loss of function of citrin, and changes that also occur clinically.
We have also considered using clinical samples, collected from the cohort of patients connected via the Citrin Foundation.

Why were they not suitable?

We have currently been utilising established liver cell lines and using gene editing approaches to generate cell culture models for citrin deficiency. However, there are limitations with the cell culture models available, even for just studying the changes in the liver cells, which is the primary organ affected by the disease:

- Established cell lines have multiple copies of genetic material (more than 2 copies of each gene). This means that the genetic editing we introduce does not accurately reflect what is happening in a human patient. Furthermore, the different numbers of genes (resulting from multiple copies of DNA) means that the cell line metabolism is very different to that of a liver cell, and thus does not give us a "true" picture of what is going on in the disease.

- We cannot "see" the different stages of the disease in cell culture models. We can measure how metabolism, cell stress and cell growth change over time, and how potential treatments work directly onto genetically altered liver cells, but cannot relate this back to the different stages of the human disease.

- Whilst the liver is the primarily affected tissue, the disease affects the whole body, and we need to understand the interplay between what is happening in different organ compartments, and systemically (i.e. in the blood). We cannot do this using cell culture as we only have one cell type, in a dish, not communicating with the other tissues of the body. Moreover, there are likely to be regional variations within any given organ, such as the liver, in how it is affected by the disease as it is progresses. These regional differences are not able to be observed, or studied without the whole organ.

We will continue to utilise our cell culture models, alongside the animals in this project, to further our understanding of the cellular mechanisms at play in the disease. They will allow us to interrogate possible metabolic pathways that may be altered in detail, following on from the changes we detect in the animal models, but we need to observe the changes first in an animal model of the condition.

Where possible we will utilise clinical samples in our work. However, given the wide range of mutations that cause citrin deficiency (more than 100), and the relatively small number of identified patients worldwide (approximately 1000 known to the Citrin Foundation, although the prevalence is relatively high in East Asia), there is expected to be a fair amount of variation in samples collected which would make establishing a how the mutations cause the clinical symptoms shown very challenging. We would need to carry

out carefully controlled experiments in animal models to understand the causal relationship before using clinical samples for validation of our findings.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Our aims fall in two parts, (a) validation of a rodent model of citrin deficiency and (b) utilising a valid model of citrin deficiency to understand the molecular mechanisms causing the disease and testing potential treatments.

For the validation aspect of the project, we anticipate we will need to generate 4-8 cohorts of different ages of each proposed model of citrin deficiency. There are currently four commercially available models of citrin deficiency, and breeding colonies will need to be established to ensure we can have age-matched controls (animals without the mutations in the citrin gene, that have been bred and raised in the same way so provide a comparative baseline group to compare the citrin deficiency model animals to). Previous work has indicated that between 6-10 animals per group is sufficient to indicate if there are differences in groups. We may need to generate a new model, e.g. an inducible model or liver-specific knock out, and thus also require animals for generating these new animals. We therefore anticipate utilising 310 rats and 420 mice for validation and generation of new models.

For the second aspect of the project, we estimate we will run around 10-15 individual experiments, each with between 2 and 8 groups. With our previous work indicating that 6-10 animals would be sufficient per group, this indicated that we would anticipate utilising 800 animals' total.

We will also need to establish breeding colonies for the mice and rats. As only half of each litter will initially be utilised for experiments (those animals carrying the mutation in the citrin gene [the 'knock- outs'] and those without the mutations ['wild types']), we anticipate needing breeding colonies of 900 rats and 1400 mice across the two breeding protocols, of which half the animals will be maintained on these protocols only.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

During the design of individual experiments, we ensure that we include appropriate controls to address our particular scientific question and utilise statistical analysis to ensure an appropriate number of animals are used. In order to minimise animal numbers and ensure randomisation and blinding where possible, we will utilise the NC3Rs Experimental Design Assistant to help design individual experimental set ups.
Whilst much of our work will focus on the liver, the primary disease affected by citrin deficiency, we will take a systems level approach (looking across the whole body rather

than one tissue) when establishing individual experiments, involving either studying or banking tissue across organ systems for later analysis. This ensures that, not only do we get a more complete understanding of how the disease impacts the whole body, we have 'pre-collected' tissue from previous studies for follow up analysis, and thus don't need to use more animals for future work.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Whilst we appreciate that we are likely to need to set up our own breeding colonies in order to study the animals throughout their lives, we will utilise the resources on efficient breeding available from the NC3Rs to ensure that animals are not wasted e.g. by using intermittent breeding methods. Where possible, we will buy in animals from a commercial supplier instead of setting up a breeding colony, to ensure breeding is only established when absolutely necessary. We will also follow best practice with regards to archiving strains, to ensure that constant breeding is not required.

The initial phase of our project aims to validate a rodent model of citrin deficiency. Whilst various approaches to generate animal models of the disease have been taken in the past, there has been no systemic, organised study of whether these models do, in fact, model the condition. Through the initial phase of the project, we aim to have a valid rodent model for the condition, which will enable us to reduce the number of animals required in the later stage (understanding the mechanism and testing therapies) as only the validated model of the condition will be required. This will also help reduce numbers of animals utilised more generally beyond this project, as results obtained will be more reproducible once in a validated animal model of the condition.

Pilot studies will be used in order to minimise the number of total experimental groups needed to answer particular experimental questions. This will also enable us to also optimise experimental conditions and refine the methodology we use in our experiments. For example, we may need to intervene with dietary modification in these animals, as in the human condition, to minimise their carbohydrate intake, and a small pilot study will determine whether this is necessary, before we move on to studying a full cohort of animals throughout their lifetime.

At post mortem, all tissues are routinely stored to ensure that the minimum number of animals are used across the programme of work. Whilst the disease of interest primarily affects the liver, we will routinely collect tissues across the body, both to enable a more complete understanding of the disease, and to reduce the number of animals that may be required in future studies. This strategy has been of great success in the license holders previous laboratory, where six peer-reviewed papers (thus far) have been published from banked tissue from a single-mouse study, initially carried out in 2012.

We also happily take part in the sharing of tissue, both for optimisation purposes and with collaborators for experimental purposes. When we need to optimise a particular experimental procedure ex vivo, we work to utilise tissue that has already been collected for another experiment but is in excess, so no animals need to be killed specifically for optimisation purposes. We likewise extend this courtesy to others. In order to do so we are already part of internal tissue sharing and colony management resources, utilised for this purpose. We have also utilised tissue collection from animals held within a commercial supplier, to enable us to begin validation of the animal model, in concert with another lab in

Switzerland, to enable us to establish maximal data about the animal models prior to having any animal experiments take place.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will be utilising genetically altered rats and mice which will not express the mitochondrial protein citrin. Whilst we will be aiming to model and characterise an animal model of the human disease citrin deficiency, for most of these animals' lives, we expect them to experience no more than perhaps some dietary changes, and thus no lasting harm. If some of the animals experience more symptoms associated with citrin deficiency, this may cause some fatigue and some changes in behaviour, which may be accompanied with some signs of liver dysfunction (jaundice) but will not be severe.

In some cases, we may feed animals a different diet or administer a compound via the animal's diet or drinking water. This may be to develop a particular clinical symptom, or to test a potential treatment. In all cases, the food/water will remain palatable to the animals, so they will continue to eat and drink. Providing an intervention in this way allows us to non-invasively administer our desired compound (or altered diet) to the animals, and therefore reduces the distress associated with alternative methods, such as direct feeding into the stomach (oral gavage) or injections. However, occasionally, in order to give a specific dose of a compound (e.g. a potential therapeutic drug), we may need to provide it via the oral gavage or injection route. When this is required, we will utilise the route of delivery that is both optimal for the efficacy of the compound, but also allows the compound to be delivered in the most pain-free way. To ensure this, we will carefully way-up the volume required, viscosity of the injection and how many injections would be needed, to enable compounds to be delivered in a manner that causes the least pain, suffering and distress. For all injections, single use needles will be used, and sterility would be ensured to prevent infection.

On occasion, animals will need to be single housed. This may be for behavioural testing (see below) or for measuring whole body oxygen consumption in "metabolic cages". Single housing the animals can be distressing for them, but we will aim to minimise this by (a) only carrying out these tests when it is the only possible technique to achieve the scientific aims (therefore minimising the number of animals that experience this); (b) keeping the time for single housing as short as possible; (c) housing single- housed cage-mates near each other, so they can still smell each other, as we have found previously this helps minimise the distress associated with single housing, and; (d) wherever possible, rehousing animals into group cages following the period in single housing. By following these optimisations, we are able to minimise the distress associated with any period of single housing.

Much of our work relies on monitoring changes to normal body functions that occur as a result of the genetic mutation in the animals. Some animals may undergo non-invasive imaging, which require temporary restraining of animals or brief anaesthetic. The scans themselves are painless and cause no lasting harm, and allow us the measure and follow many changes in how the animals are functioning without having to undertake much more invasive procedures such as tissue biopsies. Whilst restraining the animals can cause transient distress, the scans on conscious animals are very quick (less than 2 minutes) minimising this stress. Anaesthetic is only used when we need the animals to be still for longer periods of time for imaging (less than 1 hour) and although animals will feel groggy on recovering from anaesthetic, it is well tolerated in rats and mice and causes no lasting harm.

We will sometimes need to take blood samples from animals. Whilst the act of having small amounts of blood taking may cause a small amount of distress or pain, there is no lasting suffering or harm. We will utilise the most up-to-date, refined procedures for blood sampling, including taking blood from the leg or tail vein and “micro-sampling” to take minimal blood samples from each animal. In this way, we can ensure that we are causing the minimal distress possible during the procedure itself.

Some animals may need to undergo tests to assess their exercise capacity. We are utilising a methodology that is optimised to minimise distress for the animals which undergo these tests. Usually, animals undergoing exercise testing are required to be exposed to averse stimuli (shock grid, air puffs) throughout the testing, with testing only ceasing when animals remain stationary for a prolonged period of time despite continuous exposure to the stimulus. Whilst we are using the more refined air puff stimulus, the continuous exposure can remain distressing for animals. Our applied method instead requires animals to only be exposed to air puffs during training on the equipment. The test instead stops when the animal spends time within a set distance of the end of the treadmill and cannot be encouraged (by the researcher or an air puff) to continue. Animals therefore do not experience the continuous air puffs that normally end a classic test, therby reducing the distress. Animals participating will recover fully from the training and actual testing procedure and therefore not experience any lasting harm.

Likewise, some animals may undergo behavioural testing. Where possible, these will be “home cage tests” where we can assess behaviour either directly in the animal’s home cage, or in an identical environment to the animal’s home cage (but with a single animal). In these cases, the animal has very little idea that any testing is occurring, and thus there is little if any distress associated with the experiments. In other tests, we will undertake behavioural tests that are well established in psychology and neurobiology. In order for us to get usable data out of these tests, we need the animal’s performance in the test to reflect their actual behaviour, and not any distress associated with the testing equipment. We therefore will utilise well established protocols for acclimatising and training animals to all the testing equipment prior to any testing. This enables us to ensure that any distress is minimised. The tests themselves are not painful and cause no lasting distress or harm.

Why can’t you use animals that are less sentient?

Mice and rats are the least sentient animals that are suitable for studies of mammalian metabolism. Most non-mammalian organism that possesses a citrin gene, e.g. Zebrafish, have copies of the gene that is very different to the human version, and resembles a

protein that is not found in the organs affected by citrin deficiency. Less sentient animals, such as flies and worms, actually do not have a citrin gene at all and therefore are unsuitable models for us to utilise for trying to model the condition. We are therefore unable to use species that are of a lower neurophysiological sensitivity to study the metabolic physiological alterations occurring in a disease such as citrin deficiency. Given the complex way citrin deficiency presents, with separate clinical conditions occurring in infants and older people, we need to study animals across their lifespans. Metabolism also alters over development, so the interaction that mutations in the citrin protein have with these developmental metabolic changes are important for us to measure, in order to gain a better understanding of the disease progression and how new therapies could be developed. Whilst some experiments will involve animals at an immature life stage, we will need to follow animals to adulthood and as they age, in order to gain a greater understanding of the disease progression.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In all studies, animals will be monitored frequently. Our work relies on us detecting potentially very small changes in the animals with different genes, so animals will be very closely monitored throughout their lives. More often than not this will include weekly measurement of body weight, with cage food and water intake likely measured more frequently (e.g. 2-3 times a week). This ensures that animals become habituated to the researchers handling them, reducing any stress associated with this husbandry task. We also employ the use of welfare scoring systems to ensure that there is a standardised measure of picking up subtle changes in animal welfare across different researchers that may be checking the animals.

We utilise the most refined methods for handling animals, such as cupping and tunnel handling, which minimises any stress associated with handling procedures. Animal home cages also will contain environmental enrichment (to the extent at which they will not interfere with scientific studies) including, but not limited to, comfortable nesting material, shelters, play tunnels and gnawing blocks, to further optimise welfare. Any animal purchased from a commercial supplier (as opposed to being bred in the animal facility) will undergo an acclimatisation period of at least 7 days following arrival in the facility before they are used for any breeding or experimental protocol, to allow them to adjust to the new environment and recover from any distress associated with the transit.
Animals that may need to be restrained, e.g. for imaging or for blood taking, can also be introduced to the restraining tubes by the researchers during their frequent interactions with the animals during a study, prior to any procedure being carried out. This ensures that animal is familiar with the restraining devices, and observes minimal stress when the procedures (e.g. non-invasive imaging or blood sampling) are ultimately carried out. Where possible, restraining tubes will also be made of a red plastic to further minimise the distress to the animals, as it minimises the light they detect, and is more comforting for them. Any experiment that involves needle use (blood sampling, drug delivery, etc.) will utilise single use needle protocols, and needles will be the appropriate size (gauge and length) for the animal in question and route of delivery.

In the animal facility, animals will be maintained on regular light/dark cycles (12h/12h) in a temperature and humidity-controlled environment. If for some reason, the animals need to change location (e.g. for monitoring their whole-body oxygen consumption rate for measuring metabolic rate), the environmental conditions will be as closely matched to that

of their home cage environment as possible. The exception to this may be the temperature, as for some metabolic rate measurements, the animals may be maintained at a temperature closer to that at which they don't have to use any energy to maintain core body temperature (the thermoneutral temperature).

Whilst animals may have to be single-housed, for example as in the metabolic rate experiments above, this will be undertaken as infrequently as possible, and for the shortest possible time. Animals will also be kept in proximity to their cage-mates, as previous experience has shown that this reduces the stress they experience during the period of single-housing, and often allows us to return the animals to group housing afterwards. Any experiment that requires more advance equipment e.g. behavioural testing or exercise testing, will always be preceded by either a training period or, if this is not possible due to the specific scientific question, an acclimatisation period, where the animal is able to experience the equipment and get used to the new environment without any data being collected. This is also important to us, as it ensures that the results we collect are valid and reflect an actual change in the animal's physiology, rather than a change resulting from generic acute stress due to a new situation.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Experiments will be planned in line with PREPARE1 guidelines and ARRIVE2 guidelines (v2) will be used when reporting studies.
Guidance of the most refined techniques for, for example, handling, blood sampling and enrichment, from the NC3Rs resource library3 will be followed, and regularly reviewed to ensure it is up to date.

Guiding principles from the Laboratory Animal Science Associate (LASA) will be followed with respect to record keeping4, mortality reduction5 and best practice in behavioural aspects6 of our work. We also will carry out all genetic alteration experiments in line with the LASA position paper on transgenics7.
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[1] Smith AJ et al. PREPARE: guidelines for planning animal research and testing. Laboratory Animals. 2018;52(2):135-141. doi:10.1177/0023677217724823
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[7] LASA Position Paper: Transgenics. Accessible at: https://www.lasa.co.uk/wp-content/uploads/2018/05/Position_Transgenics.pdf

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The license holder receives monthly updates from the NC3Rs to remain up-to-date with current advances in 3Rs research. The license holder also subscribes the Tech3Rs specialist bulletin to receive current information relevant to 3Rs practice in husbandry and care e.g. improved handling, blood sampling etc. We also receive information on relevant courses from the Named Information Officer of the Establishment. We work closely with an experienced laboratory animal models manager, which enables a large wealth of experience to be disseminated effectively to our group.
The small number of researchers working under the project enable these advances, which are often easy to implement, can quickly be passed from one member of the team to the whole group.

Discussion and feedback within the team is welcome allowing new techniques and advances in the 3Rs to be implemented effectively throughout the project for the positive benefit of the animals.

97. Therapeutic targeting of KRAS-mutant lung cancer and small cell lung cancer

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Lung Cancer, Metastasis

Animal types	Life stages
Mice	Adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To identify changes in lung cancer cells that relate to their ability to multiply and spread. Ultimately to use this knowledge to develop new treatments for patients with lung cancer. Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Lung cancer accounts for nearly a quarter of UK cancer deaths. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of lung cancer cases and its most common growth pattern is adenocarcinoma, a type of cancer that starts in the glands that line internal organs (around 40% of lung cancer patients). The most common (~30%) driver

mutations for lung adenocarcinoma are in a gene called KRAS. Genetic data has altered the classification of NSCLC over the past 10 years, highlighting specific genetic alterations which are susceptible to targeted therapies. These drugs have improved outcomes of patients carrying these genetic alterations. Even though for years it was thought that KRAS could not be targeted by drugs, in recent years drugs for two common KRAS mutations, KRAS G12C and KRAS G12D have been developed. However, the effectiveness of these drugs is limited by several resistance mechanisms. Moreover, different mutations in the KRAS gene may promote lung cancer formation via different mechanisms and this may be exploited for mutation- specific treatment. We therefore aim to characterise the relative roles of the different KRAS mutations in NSCLC development using cell-based, non-animal, as well as mouse studies. In doing this, our goal is to support the development of personalised medicine for NSCLC.

Another type of lung cancer we are investigating is small cell lung cancer (SCLC) which accounts for approximately 15% of lung cancer cases. SCLC is a highly aggressive cancer associated with poor prognosis. Its aggressiveness is linked to the presence of two different cell types within SCLC tumours, a phenomenon known as intra-tumoral heterogeneity. This diversity arises from the ability of tumour cells to transition from a cell type called neuroendocrine (NE) to one called non-neuroendocrine (non- NE). In mouse models, the coexistence of both NE and non-NE cells has been linked to the spread of cancer to other organs (metastasis), a challenging clinical problem and the cause of most cancer deaths, as well as resistance to treatment. As such, understanding the mechanisms driving this transition is important for the development of future therapeutic approaches. Our proposed project aims to use cell-based, non-animal experiments as well as mouse studies to understand the triggers promoting this transition. We also aim to again use similar studies to understand the molecular mechanisms (other than NE to non-NE transition) promoting the movement and spread of SCLC cells.

What outputs do you think you will see at the end of this project?

The primary output of this project is data/information that advances our mechanistic knowledge of how different mutant variations of KRAS drive the development and progression of lung adenocarcinoma. We are also going to gain an understanding of the mechanisms driving neuroendocrine (NE) to non- neuroendocrine (non-NE) transition of small cell lung cancer (SCLC), which is strongly linked to metastasis and resistance to treatment. Moreover, we aim to understand the molecular mechanisms (other than NE to non-NE transition) promoting the movement and spread of SCLC cells. Our findings will be made available to other scientists through publication in peer-reviewed journals and presentations at scientific conferences and meetings.

The expected benefits of the work can be summarised as follows:

1) Knowledge of the potential of different KRAS variants to promote the development of lung adenocarcinoma and the pathways by which they cause NSCLC tumour formation and progression
2) Understanding of the mechanisms promoting SCLC heterogeneity
3) Understanding the molecular mechanisms (other than NE to non-NE transition) promoting the movement and spread of SCLC cells
4) By integrating the knowledge gained from the above research, we aim to identify key therapeutic targets for future hypothesis-driven therapeutic intervention

5) Test novel therapeutic agents currently investigated in clinical trials and their potential mechanism of loss of effectiveness due to drug resistance
6) Publication in high-quality journals and presentation at conferences to share the work with the wider scientific community

Who or what will benefit from these outputs, and how?

We anticipate that a large part of benefits stated above would be seen within the 5-year duration of the project. The main beneficiaries of these will be other scientists, health professionals as well as pharmaceutical companies working on lung cancer. We anticipate that patients won't be able to benefit from this work within the time frame of this project licence, but might benefit in the next 10-15 years.
Patient benefit might be via better understanding and classifying lung cancer so that we identify which patients are likely to respond to current treatments as well as by developing new compounds.

How will you look to maximise the outputs of this work?

Our findings will be made available to other scientists through collaborations, publication in high-quality journals and presentations at scientific conferences and meetings. Our Establishment and funders have a policy of ensuring that all publications from their scientists are available on free access to all.

Species and numbers of animals expected to be used

- Mice: 1500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are more comparable to humans than other animals (e.g. fish, insects) in how their organs function and how disease develops. Also, they show higher similarity of protein sequences. This is important as we intend to use reagents such as small molecule drugs and antibodies that have been developed to target human proteins.
Moreover, non-protected species and less sentient species (e.g. fish, insects) do not have lungs so we would be unable to use them for animal models of lung cancer. Embryonic stages would not provide us with a sufficient window to follow tumour development and besides it is not feasible to perform the desired interventions in embryos (such as inhalation of activating agents). Therefore, adult mice are to be used.

Typically, what will be done to an animal used in your project?

Some mice (bred under other Project Licences) harbour genetic modifications that predispose them to the development of lung cancers when exposed to an appropriate inducing agent. Furthermore, some genetic modifications allow us to track specific cells within the tumour (such as T cells, type of white blood cell). In other mice, which are bred specifically to tolerate human cells (without cell rejection), tumours will be grown under the

skin of mice for ease of monitoring or into the lung so that cells are placed in their normal physiological environment. Mouse cancer cell lines can be transplanted into mice sharing the same genetic background (so called syngeneic mice) without rejection. Tumour growth is typically not associated with pain during the period in which we conduct our observations.

However, occasionally tumours might become ulcerated. Animals with ulcerated tumours will be closely monitored and managed according to best practices at our Establishment. If these tumours start bleeding and become sore, then animals will be humanely killed. Tumour growth will be monitored regularly by either use of callipers for tumours grown under the skin of mice, or by imaging methods for internal tumours. Mice with tumours will be monitored daily. For procedures that involve surgery, such as implanting human tumour fragments or removing a primary tumour in order for a secondary tumour to grow, which involves fewer than 10% of mice, we will administer painkillers around the surgery period and monitor the mice closely during recovery from general anaesthesia.
Around 25% of mice with implanted tumours may have either potential novel therapeutic agents, existing clinical agents or placebo, administered by a variety of routes, but usually either by mouth, or by injection either under the skin or into the abdomen to study the effects on tumour growth and/or tumour composition. Mice may be studied for up to 200 days after a period of therapeutic agent treatment for tumour growth. The mice might also have blood samples taken either from the tail vein or by sampling from a heart chamber under anaesthesia (in which case the animal does not regain consciousness before being humane killed). Occasionally mice may be administered organ preservatives whilst under non-recovery anaesthesia to allow us to undertake investigations on slices of selected organs observed under a microscope.

Mice will be group housed in ventilated cages which have their environment enhanced with items such as tunnels, nesting material and gnawing blocks.

At the end of any protocol mice will be humanely killed.

What are the expected impacts and/or adverse effects for the animals during your project?

The mutations which predispose to cancer are not expected to cause any adverse effects per se as these in most cases only manifest following inhalation of inducing agents. Following inhalation of inducing agents, mice carrying mutations predisposing them to cancer are expected to have lung tumours. It is possible that the tumour growth might affect normal physiological functions (such as eating, locomotion or breathing). However, mice will be observed daily and any side effect that cannot be managed satisfactorily will result in humane killing of the animal to avoid pain, distress or suffering. The growth of implanted tumours is limited to avoid serious complications. However, occasionally tumours might become ulcerated. Animals with ulcerated tumours will be closely monitored and managed according to best practices at our Establishment. If these tumours start bleeding and become sore, then animals will be humanely killed.
Injections would only cause very transient pain.

After surgical procedures we will monitor mice for signs of pain and administer effective pain relief for as long as it is required.

Drugs will be used at non-toxic doses and administration by injection is only minimally invasive. Oral administration can be unpleasant for mice and carries a risk of injury, but this rarely happens as it is performed by experienced personnel.
Imaging of mice is performed under general anaesthesia from which they awake. It happens only rarely that mice do not recover from anaesthesia and need to be killed. Otherwise, general anaesthesia is not harmful or painful.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The vast majority of mice are only expected to experience the mildest clinical symptoms due to tumour growth before they are humanely killed. Additionally, some mice will experience the discomfort of repeated (daily) injections of therapeutic agents or oral delivery with a specialist tube. We will aim to utilise the least stressful route of administration wherever possible. Overall, we expect ~85% of the mice to experience Moderate and 15% Mild severities.
A minority of mice will undergo surgery, and these will be anaesthetised for the operation and receive painkillers post-operatively until pain subsides. Some mice will also have repeated anaesthesia for the purposes of imaging the internal tumours. Whilst loss of consciousness may be distressing this is not painful.

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

While valuable studies of human cancer are performed using tumour material and cell lines derived from both mice and human samples, the mechanistic understanding of cancer development and spread requires the use of living animals. In particular, cancer development and spread involves a plethora of interactions between cancer cells and their surrounding host and their behaviour is governed by multiple signals originating from both their immediate neighbours and from distant tissues.

Genetically altered mouse models have been engineered to develop cancers, which accurately mimic their human counterparts. These models can be used to test the effectiveness of novel cancer therapeutics that have been developed for humans as there is great similarity between human and mouse proteins. Mice cannot be replaced by different animal models such as zebrafish or insects which remain far less complex than their mouse counterparts and for the purpose of our work do not have lungs.
Searching the scientific literature in MEDLINE using specific keywords indicates that no comparable studies has been performed in mice.

Which non-animal alternatives did you consider for use in this project?

We routinely use several NSCLC and SCLC cell models (such as H358, H441, H1299, A549, H2171, H526, H146, DMS53) included in our previous publications and patient-derived primary cultures of SCLC (originally grown as tumours in mice but capable of short-term growth in tissue culture). We also searched MEDLINE for "lung on a chip" to potentially use in our studies. We also make use of human lung cancer specimens and sequencing data to generate further evidence in support of our hypotheses and to check that findings are relevant to clinical samples.
The proposed research will build upon the extensive in vitro analyses performed in our laboratory which will provide preliminary evidence for in vivo experiments. In vitro studies will include measurement of proliferation, survival and invasion in the presence of drugs or other approaches that perturb gene function (RNA interference, gene over-expression). Therefore, prior to commencing mouse experiments we would be anticipating significant differences between control and test groups.

Why were they not suitable?

The study of cells in culture (in vitro) provides us with clues on the cellular processes contributing to lung cancer development in a simple and valuable context, which allows the establishment of hypotheses regarding the function of cells in a living animal. However, these systems do not recapitulate the complex cellular interactions described above. 'Lung on a chip" models have been described in the literature but currently do not incorporate the mechanical effects of breathing and lack an immune system. Sequencing data is descriptive and can only be used for corroboration not for demonstrating causality. Likewise, clinical specimens do not always mirror interventions that we are trying to evaluate (such as the acute effects of a drug) and analysis is typically confounded by multiple variables that cannot be controlled (such as diet, or comorbidity). Because of all these limitations, we still need to perform some animal experiments to progress our research.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The overall aim will be to generate models whereby a measurable effect e.g. reduction in tumour number, volume or incidence following manipulation of a gene of interest or treatment with a drug can be determined using a minimal number of animals.
Based on past experience, group sizes of between 10 and 20 animals (dependent on the readout; fewer for transplanted tumours compared to tumours arising in genetically modified mice) per experimental group suffice. However, for an experiment to be well-controlled and meaningful, we may include more than one experimental group. For instance, in implantation experiments where we deplete a gene in cells, we will use two independent reagents targeting the gene as well as a control. Moreover, we would typically

examine more than one model cell line. Likewise, we may use several doses of a drug, or several different drugs or drug combinations to test a theory. Considering power, the number of experimental groups, and the number of genes and drug targets we are interested in, we have estimated the total number of mice to be used over the licence lifetime.

Other aspects of good experimental design allow us to produce reliable data. Thus, we aim to reduce confounding factors by using mice of similar age and roughly the same weight. Also, experiments are blinded as different personnel perform the procedures from those analysing the data. Moreover, the identity of samples is withheld from the individuals analysing the data. Drug effects are controlled by contrasting with effects of a placebo, whereas genetic manipulations are controlled by engineering cell models with non-targeting modifications. Mice in a cage will undergo a range of treatments to avoid batching effects.

After data has been acquired, it is tested for normal distribution and then an appropriate statistical analysis is performed to test for significance. If the appropriate statistical analysis is not something the group performs routinely, then it will be undertaken with guidance from a statistician.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

For genetic mouse models, efficient breeding strategy will minimise the number of mice used to obtain the required experimental animals.
Experiments will be appropriately controlled, and mice of the same age, genetic background and source will be used to reduce the variability of results and to produce highly consistent data. Wherever possible and appropriate, a single group of animals will serve as a control for more than one experimental groups.

We will be conducting and recording our experiments to be able to publish our results following the ARRIVE guidelines [https://www.nc3rs.org.uk/arrive-guidelines] and will use randomisation, blinding etc. where appropriate to minimise biases. Furthermore, additional resources may be used to aid experimental design such as the NC3Rs experimental design assistant tool:

(https://www.nc3rs.org.uk/experimental-design-assistant-eda).
A statistician helped us with calculations using typical variations from our own earlier experimentation to calculate minimum numbers of animals to be used whilst ensuring that the results are statistically significant.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Pilot studies will be performed if applicable and, after analysis of the results, group sizes for subsequent experiments will be determined based upon these data. As far as possible, multiple parameters will be evaluated in a single mouse. Live imaging of the same animal at multiple time points also greatly reduces the numbers required. Multiple tissues can be extracted from the same mouse. For example, when examining metastases, we would typically remove lungs, spleen and liver.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse is the lowest vertebrate that offers an in vivo situation (anatomy, physiology, metabolism) relevant to human cancer and that can be manipulated in a manner that will generate data relevant for the treatment of human cancer. As such, the mouse is the most appropriate animal model to achieve the stated objectives. Moreover, techniques required for the proposed types of analyses are established for studies using mice making predictions of possible adverse effects more reliable.

We will use mice that have been altered genetically to either allow specific tracking of cells or which are predisposed to lung cancer conditionally upon treatment with inducing agents. Tissue-specific and inducible gene alteration will reduce the potential burden of genetic loss in the whole organism. The vast experience of animal technologists within our animal facility will be harnessed to minimize potential suffering by regular monitoring of tumour size and potential impact on the general health status of the mice. By responsibly considering the adverse effects associated with the regulated procedures, mechanisms are in place to minimise these (e.g. appropriate analgesic regimes for pain relief).
To reduce any suffering of tumour bearing mice, they will be killed humanely as soon as tumour formation is sufficient to yield satisfactory data and always before they become moribund, manifest severe pain or lose significant weight (all of which are closely monitored).

For any new procedure, we will seek expert advice and follow the most refined techniques available, experimenting at first with a limited number of mice.

For any inoculation/transplantation procedure we will follow the route that causes the minimal burden on the animal’s well-being.

Why can’t you use animals that are less sentient?

Less sentient animals do not have lungs. Mouse is far more similar to humans than other animals having lungs e.g. birds or reptiles and this is critical both for using reagents like drugs developed for human targets and for translating findings to the clinic. Furthermore, cancers develop over many weeks to months, so use of terminally anaesthetised animals or immature animals is not practicable. Also immature mice lack a functional immune system which is desirable in cancer research.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Assessment of the size of superficial tumours would usually be by callipers (usually of two diameters at right angles). The total tumour burden should not normally exceed 1200 mm3 as calculated by the formula: tumour volume = ½(length x width x 2). Subcutaneous tumours will be measured at appropriate intervals (usually once a week using callipers). If a tumour reaches approximately 2/3 of the maximum permissible volume, it will be measured more frequently.

Additionally, superficial tumours will be monitored for signs of local inflammation, irritation, or pinpoint scabbing to an ulcerated state. Animals with ulcerated tumours will be cared for according to the best practice at our Establishment. Wherever relevant, animals will be provided with analgesia as detailed in the relevant Protocol to control adverse symptoms associated with the treatment.

For non-superficial lung tumours imaging will be used to monitor the disease burden alongside clinical measures of the health status of the animals (e.g, Pulmonary Assessment of Advanced Metastasis (PAAM) to assess lung capacity along with respiratory rate and lethargy).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow guidelines of good practice [Morton et al., Lab Animals, 35(1): 1-41 (2001); Workman P, et al. British Journal of Cancer, 102:1555-77 (2010)] as templates for experimental design and decision making. Administration of substances will be undertaken using a combination of volumes, routes and frequencies that themselves will result in no more than transient discomfort and no lasting harm.
PAAM: A Novel Noninvasive Method for Evaluating Experimental Lung Metastasis in Mice (Mendoza et al, J Am Assoc Lab Anim Sci. 2013 Sep;52(5):584–589).
Guidelines for Body condition score [Ullman-Cullere, Lab Anim Sci. 1999 Jun;49(3):319-23]

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

By reading 3Rs literature and participating in 3Rs workshops locally and nationally. Through discussing refinements with our NACWO and NVS.

98. Understanding mitochondrial dysfunction in disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Mitochondria, Mitochondrial disease, Cancer, Ageing, Mechanisms

Animal types	Life stages
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult, Aged animal

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The work carried out under this license aims to;
(1) deliver new insights, therapies and biological indicators (biomarkers) of mitochondrial diseases
(2) understand the role of mitochondrial dysfunction in intestinal cancer

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Mitochondrial Disease

Mitochondria are tiny energy generators that exist in large numbers (100s-1000s) inside human cells. Mitochondrial diseases are a large group of genetic disorders affecting approximately 1 in 4300 people, that impair mitochondrial function and the body's ability to make energy. They can cause disabling symptoms that may impact very severely on quality of life. Mitochondrial diseases are caused by alterations in DNA inside the cell nucleus or a small amount of DNA (mtDNA) inside the mitochondria themselves. Mitochondrial diseases can cause symptoms at birth, in childhood or during adulthood, and cause a wide variety of medical problems, depending on the body parts most severely affected. As mitochondria are present in almost all cells, any system in the body can be affected.

Patients can develop deafness, blindness, heart problems, seizures, diabetes and gut problems among others. Muscles are particularly susceptible to reduced energy, which causes fatigue and weakness. Unfortunately, there are currently no effective treatments for mitochondrial diseases and there are a limited number of mouse models available to study mechanisms underlying these diseases, measure disease progression over time, or to test potential treatments.

Importantly, the effects of faulty mitochondria seen in mitochondrial diseases are also present in other common diseases (genetic and non-genetic), such as cancer and are also commonly detected in ageing tissues. Consequently, a better understanding of how dysfunctional mitochondria affect different cells in different tissues in the human body will help us to understand, and potentially treat both mitochondrial diseases, and other common diseases (e.g. some cancers) where mitochondrial dysfunction is a significant feature.

Colorectal cancer

Colorectal cancer is the third most commonly diagnosed cancer in men and women, with up to 42000 cases diagnosed in the UK each year, resulting in 16000 deaths per year. This is because the more advanced cancers often respond poorly to treatment. To develop better treatments which are likely to have a positive effect in patients we need to better understand a number of features of how cancers behave. These include understanding how they form, how they grow and they progress to more dangerous disease. Alterations to mitochondrial DNA are present in 60% of colorectal cancers. These can change the behaviour of the cancer cells and make them grow faster in the early stages of the disease, but we do not know their effect in the later stages of the disease and how they may alter the response of a tumour to treatment. To understand this, our study aims to develop animal models of intestinal cancer which have mitochondrial DNA alterations, meaning they will resemble human cancer as closely as possible. We will then use these to understand how mitochondrial DNA alterations affect how the cancers develop and grow, and use this to identify key new potential treatments.

What outputs do you think you will see at the end of this project?

Work conducted under this project license will generate data and new knowledge that will help advance our understanding of how mitochondrial diseases develop, and what changes at the level of the molecules and cells makes these diseases get worse over time. Our work will help advance knowledge of mitochondrial disease processes and identify

potential new drugs or druggable targets. We also aim to identify new biomarkers of disease (e.g. proteins or DNA in the blood that are released from damaged tissues) that we could use to track disease progression.

We will also generate new data and knowledge about how mitochondrial dysfunction affects intestinal cancer development and advancement of the tumour. We aim to understand how mitochondrial dysfunction affects the cancer cells and the molecules within them so that we can both identify new drug targets, and test their sensitivity to existing intestinal cancer treatments.

Our work will be presented at scientific meetings and published in scientific journals to share the knowledge that we gain with the wider scientific community. This will help to advance knowledge in the field for the ultimate benefit of patients.

Who or what will benefit from these outputs, and how?

In the short term, scientists in both academia and industry will benefit from the discoveries generated under this programme of work. This could be due to the development of new research tools, experimental approaches, or identification of new pathways which, when targeted, yield therapeutic benefit.

The long-term aim is to benefit patients either through the development of new biomarkers or new treatment strategies.

How will you look to maximise the outputs of this work?

By presenting our discoveries at national and international scientific meetings, publishing our research discoveries, and through collaborations with academics or the pharmaceutical industry, we will be able to maximise the impact of knowledge gained under this programme of work.

Wherever possible we collaborate with others to share tissue samples, cell or mouse lines, and provide training in methods through collaborative research or participation in workshops.

Species and numbers of animals expected to be used

- Mice: 12000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use mice because they are powerful tools for understanding human diseases and how they progress. They share 99% of the human genome and many anatomic and physiological similarities with humans. They are mammals, and therefore have specialised organs, unlike lower organisms. We need to use genetically modified mice to study

disease biology, and ask how a genetic alteration which has been identified in patients to increase the risk of developing a disease, affects the disease process.

We can genetically modify the mouse genome to generate mammalian models in which to study mitochondrial diseases and intestinal cancer. Mitochondrial diseases and intestinal cancers develop over many weeks/years so we need to model the disease process across the whole lifespan, therefore juvenile, adult and aged animals may be used. We need to do these experiments in whole animals as the diseases we are studying often affect multiple organ systems within the body, and we need to be able to investigate the interplay between the different organs. We have performed a comprehensive search of the literature and found that there are currently no human or mouse cell models (in vitro models, which are studies that are performed with microorganisms, cells, or biological molecules outside of a living organism) capable of a comprehensive study of the mitochondrial dysfunction across multiple, interacting physiological systems. We cannot use less sentient animals such as insects or fish as their nervous system does not work in the same way and they lack some of the immune cells present in humans, meaning that they cannot model the diseases that we are interested in accurately.

Typically, what will be done to an animal used in your project?

Mitochondrial Disease

Many of the mice on this licence will be used only for breeding (~80%). This is due to the need to carry out several steps of breeding to obtain a suitably sized cohort that can be studied . Mice with the correct genotype may undergo some of the steps detailed below. Mice without the desired genotype will be humanely killed. Wherever possible tissues from these mice will be shared with other researchers to maximise animal use.

Some animals in this project may undergo injection of chemical and/or biological substances to investigate how the disease is affecting the cells and organs (~10%). Some mice may be administered with therapeutic agents either by injection, or in their food or drinking water to see if this improves their disease symptoms. Some of these may be novel therapies administered over weeks or months (~5%).

Mice may be fed a modified diet for (over weeks or months) (e.g. high fat) with or without sugar water (comparable to coke) to see how this affects their disease symptoms (~5%).

We may wish to perform non-invasive imaging whilst the animals are asleep, assess well-being using behavioural tests or measure blood pressure (~5%). We may take blood samples to assess disease stage or drug metabolism and we might perform glucose tolerance tests (~5%). These experiments will be performed at defined intervals over the lifetime of the mouse (up to 2 years)

Animals may undergo a combination of both substance administration combined with behavioural testing (~5%).

Intestinal cancer

Many of the mice on this licence will be used only for breeding (~80%). This is due to the need to carry out several steps of breeding to obtain a suitably sized cohort that can be studied . Mice with the correct genotype may undergo some of the steps detailed below.

Mice without the desired genotype will be humanely killed. Wherever possible tissues from these mice will be shared with other researchers to maximise animal use.

In our genetic models of intestinal cancer the tumours may need to be induced by injection of a chemical into the mouse's abdomen, or by an injection into the colon wall using a colonoscope while the mouse is asleep under anaesthesia. The cancers will develop over a number of weeks or months (~5%).

In some mice tumour cells will be implanted into the colon using a colonoscope-guided injection whilst the mouse is asleep under anaesthesia. The cancers will then develop over a number of weeks or months (~5%).

Some animals in this project may undergo injection of chemical and/or biological substances to investigate the biology of the tumour (~5%).
Some mice may be administered with therapeutic agents either by injection, or in their food or drinking water so see if this reduces the tumour growth over weeks or months. Some of these may be novel therapies (~5%).

Some mice may undergo targeted radiotherapy (a treatment where radiation is used to kill cancer cells) to see if this reduces the tumour size/growth (~5%)
Growth and development of tumours may be monitored using medical imaging techniques such as Magnetic Resonance Imaging (MRI), Computed Tomography (CT) ultra sound, colonoscopy etc. (~5%). These experiments will be performed at defined intervals over the lifetime of the mouse (up to 2 years).

We may assess well-being using behavioural tests or measure blood pressure (~5%). We may take blood samples to assess disease stage or drug metabolism and we might perform glucose tolerance tests (~5%). These experiments will be performed at defined intervals over the lifetime of the mouse (up to 2 years).

What are the expected impacts and/or adverse effects for the animals during your project?

In our mouse models, mice may show signs of sickness e.g. hunched posture, diarrhoea, ruffled fur, or look pale. Mice may show some or all of these symptoms to varying degrees. We will closely monitor all of the mice using a score sheet and give supportive care if required. Any mouse which reaches our pre-defined endpoints e.g. if it loses 20% of its body weight, will be humanely killed. We expect the duration of these symptoms of sickness occur to be less than a week.

Mitochondrial disease models may develop symptoms such as weight loss, muscle weakness, tremor or anaemia. We expect these symptoms will develop very slowly over the lifetime of the mouse. We will not allow these mice to age beyond 26 months. Mice may show some or all of these symptoms to varying degrees, therefore we will closely monitor all of the mice using a score sheet and give supportive care if required. Any mouse which reaches our pre-defined endpoints e.g. if it loses 20% of its body weight, will be humanely killed.

Intestinal cancer models will develop tumours over a number of months, predominantly in the intestine. The tumours may spread to other organs in a small proportion of the mice (~10%). As the tumours get bigger, the mice may show other symptoms, predominantly

weight loss and anaemia (a condition that develops when your blood produces a lower-than-normal amount of healthy red blood cells. If you have anaemia, your body does not get enough oxygen-rich blood that can make you feel tired or weak). Mice will be monitored using a clinical score sheet. Any mouse which exhibits clinical signs indicating that their condition is deteriorating e.g. more than 20% weight loss or marked anaemia (where the blood has a lower-than-normal amount of red blood cells) will be humanely killed.

In both mitochondrial disease and intestinal cancer models we may age some of the mice to reflect the human disease. Therefore those mice may develop age-related effects such as; weight loss, greying of hair or alopecia, ruffled fur, ocular deterioration (eye cloudiness or cataract formation) reduced activity, reduced subcutaneous fat and signs of kyphosis (curving of the spine). Mice may show some or all of these symptoms to varying degrees, therefore we will closely monitor all of the mice using a score sheet and give supportive care if required. Any mouse which reaches our pre-defined endpoints e.g. if it loses 20% of its body weight, will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Only mice will be used in this program of work.
We estimate that ~80% animals used in procedures will be mild including breeding mice to maintain a colony, for experiments and to take tissue.

We expect ~20% of the mice will experience moderate severity either because they will develop tumours or they will experience a combination of procedures such as genetic alteration combined with administration of therapy, blood sampling and imaging.

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

<u>Mitochondrial Disease</u>

Animal models are required for the study of mitochondrial diseases because they are highly complex disorders, often affect multiple organ systems within the body, and can result in different clinical phenotypes. For the majority of mouse models we use, we will be studying multiple systems involving many organs of the body, and the interplay between them (e.g. behaviour and metabolism). There is currently no human or mouse cell models (in vitro models) capable of a comprehensive study of the mitochondrial dysfunction across multiple, interacting physiological systems. We also want to test potential therapies to

improve mitochondrial function. It is important that this is done in live animals to test the effects of medicines on the whole body. This is both in terms of how the body takes in the medicine, how the medicine moves around the body and how the body get rids of the medicine (pharmacokinetics), and what the medicine does to the cells (pharmacodynamics). This cannot be predicted in human tissue/cells or cell culture systems because of the complex nature of the way the body's systems work together.

Intestinal cancer

Cancer is a complex disease, which also involves multiple cell types. For example, the way a tumour grows can be affected by the numbers and types of immune cells, which are present in the place in the body in which it is growing. We wish to understand how mitochondrial dysfunction not only influences growth of the initial tumour, but also how it affect the way that tumours spread from the intestine to other parts of the body such as the liver or the lung. It is only possible to study this in the whole animal. We also want to test new medicines to treat cancers, which requires live animals for the same reasons as stated above for mitochondrial diseases.

Which non-animal alternatives did you consider for use in this project?

Wherever possible, we will use human tissue/cells or cell culture systems to replace animal models of mitochondrial dysfunction and cancer. We have accumulated archival tissue banks of frozen and formalin fixed tissues from our previous models and from healthy and diseased human tissue. These samples are used in multiple ongoing projects to minimise the number of animal models used.

We routinely use human cells in culture (including cell lines) to understand and model the biological processes affected by mitochondrial dysfunction both in normal and cancerous cells and to perform initial drug testing.

We have started developing 3D culture systems as an alternative method for drug screening and to understand the effect of mitochondrial dysfunction on cancer cells. We are also using co-cultures of human neurons and inflammatory cells to model how mitochondrial dysfunction affects the interplay between different cell types. These advances will help to minimise animal use.

Why were they not suitable?

Whilst these are useful tools, there are limitations of cell cultures systems, these include;

1. Cells grown in culture need a plentiful supply of nutrients, particularly glucose. This means that they are often not reliant on normal mitochondria to function and therefore when they have mitochondrial dysfunction they do not behave in the same way that they do in the body. These abnormal behaviours could lead to the identification of non-relevant pathways or fail to predict drugs that are likely to be ineffective in the disease.
2. Cells grown in petri-dishes sit on plastic, which changes their biological characteristics and they become "super sensitive" or fail to recapitulate their normal biological role.
3. The in vitro screen and systems we use prior to animal studies are extremely useful for aiding our basic knowledge and for initial drug screening, and we use these systems to reduce animal use however, they cannot fully recapitulate the behaviour of a drug in a complex biological system

4. Whole-body work is required to understand mitochondrial disease pathology, as there is evidence for the interplay of between different tissue/organ systems. For example, patients with a mitochondrial disease called Leigh syndrome have a defined type of brain abnormality. The mouse model recapitulates these lesions quite closely. However, it has been shown that cell culture and brain organoid models do not recapitulate the types of brain abnormalities observed and do not respond to interventions that were successful in the mouse due to a lack of immune cells which are cells necessary to drive the disease, thus limiting their use in pre-clinical investigation. As organoid systems become more advanced, this may become possible, but technology is currently lacking for these applications.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have carefully designed our breeding strategy to make sure that we generate the most animals possible with the correct genetic alteration and the fewest with the incorrect genetic alteration. The breeding strategy has been designed in consultation with our collaborators who have extensive experience with the models we will use. In addition, we will work with our colony management team and follow local guidelines to preserve colony health, monitor any unexpected genetic defects and ensure preservation of the genetically altered lines. These refinements include:

1. Backcrossing of mouse strains at appropriate points such as every 5-10 generations, dependent on colony size. This will preserve genetic health and increase reproducibility by limiting genetic drift that occurs naturally across generations.
2. Utilising analysis tools such as Single Nucleotide Polymorphism (SNP) analysis, to ensure correct back crossing to preserve genetic health. Analysis will occur either when embryos are frozen and/or before backcrossing occurs to establish the correct choice for background.
3. Utilising the breeding system that is most suited to the efficient production of each individual strain of mouse, this will be based on data or information available in mouse passports e.g. pairs/trios.

Breeding of mice with changes to their mitochondrial DNA (mtDNA) poses different challenges when compared to mice with changes to their nuclear DNA. For example; mtDNA is only inherited through the maternal line. A certain level of the mtDNA mutation (usually over 70%) is required before mice show a biochemical or clinical phenotype; mtDNA randomly segregates into egg cells (oocytes) meaning that a female with 75% mutated mtDNA may produce individual egg cells (and therefore offspring) which contain anything from 40-80% mutated mtDNA. This means we may have to breed a larger number of animals than for nuclear mutations in order to select experimentally relevant mice carrying the required proportion of mutated mtDNA. We will avoid over supply as much as possible by carefully selecting breeding stock to increase the chances of producing experimentally relevant mice.

We have used knowledge from previous studies to mathematically calculate the minimum number of animals needed in each group to generate data, which allows us to answer our scientific questions. By doing this we can minimise use but be confident that the differences in a scientific measurement between two groups is meaningful and has not been obtained by chance. We have also consulted with our institutional statisticians (including the AWERB statistician) whilst calculating the group sizes we require. Group sizes will be larger when we need to keep mice into old age (up to 26 months). This is because, in our experience there is significant attrition (especially in males) when co-housing animals to this time point. Attrition rates are different for different background strains but we will use data from other ageing projects to ensure that the appropriate numbers for statistically and biologically relevant data will be available throughout the study

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The PREPARE guidelines were and will be referenced during the planning phase of experiments; this includes thorough examination of the literature, use of pilot studies, prevention of observer bias (by using standard operating procedures), ensuring sufficient staffing and competency is in place to conduct the selected tests, and deciding on which characteristics of the animal are essential to each specific study. Standard Operating Procedures have been written and used routinely for previous projects. This standardises the way the data and metadata is collected and reduces the variability and therefore the sample size.

In addition, constant evaluation of procedures and refinements will minimise the number of animals used in this project. Careful planning of colony expansion will ensure any surplus of animals generated is kept to a minimum. This is a particular challenge in the breeding of mitochondrial DNA mutant mice; however, the members of the team are uniquely equipped to minimise waste as they have significant experience in breeding these strains.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Statistical analysis is performed to determine the minimum numbers of animals needed to generate biologically meaningful data. If this is not possible then pilot studies are performed to reduce numbers and inform future studies going forward.
All breeding for experimental cohorts is performed according to precise breeding calculations, the formulae of which we refine and update periodically with reference to current breeding figures. We use both male and female mice to minimise numbers of animals used. The breeding strategy has been designed in consultation with our collaborators who have extensive experience with the models we will use. In addition, we will work with our colony management team and follow local guidelines to preserve colony health, monitor any unexpected genetic defects and ensure preservation of the genetically altered lines.

Tissue is regularly shared between projects to maximise outputs from animal procedures and minimise numbers of animals used.

Wherever possible, we will use human tissue/cells or cell culture systems to replace animal models of mitochondrial dysfunction and cancer. The group have accumulated

archival tissue banks of frozen and formalin fixed tissues from our previous models and these samples are used in multiple on-going projects to minimise the number of animal models used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

All of the disease models chosen are the lowest severity model that can be used to answer our research questions. Mitochondrial dysfunction can affect multiple organs of the body, therefore to

understand how mitochondrial disease develops we need to use genetically altered mice in which the mitochondrial do not work properly. We will monitor the behaviour of these mice, using minimally invasive tests wherever possible to ensure minimal suffering.
A subset of these mice will develop tumours of the intestine which we will induce via injection into the abdomen, or implantation of cells into the colon using a camera guided needle (colonoscopy). The models of cancer we will use are very well-studied, have a predictable course of disease and we are very experienced in monitoring the clinical signs of tumour development to ensure that the study is ended before the animals are experiencing unnecessary suffering or distress.

Why can’t you use animals that are less sentient?

Organs contain lots of different types of cells and these cells all talk to each other to ensure that the organ does its job properly. When cells have mitochondrial dysfunction which results in the organ not functioning properly and becoming damaged, all of the different cell types are needed to help repair the damage. Because of the complex nature of the disease process it is not possible to recreate this using cells in culture dishes, therefore we need to study disease progression and test medicines in the whole animal. The mouse is the lowest mammalian species (in terms of the sensitivity of the nervous system) in which we can perform the full range of genetic manipulations that we need to understand how mitochondrial dysfunction affects multiple organ systems, the clinical phenotype of the mouse, and intestinal cancer development. It is possible to carry out some manipulations in frogs and fish but the processes being studied here are being studied in a mammalian context and, although other animal and non-animal species can be informative in this regard, they cannot replace studies specifically in mammals. Mice remain the model of choice due to the volume of genetic information available, their relevance to humans and the relative ease of generating, establishing and preserving mouse colonies. Moreover, well-developed and characterised inbred strains of mice present an opportunity for reducing variability and therefore enhancing reproducibility. We will follow local colony management guidelines such as freezing sperm during the early

breeding steps, monitoring spontaneous genetic defects and removing non-standard mice from breeding programmes, and not breeding from animals that are older than 12 months of age. This will ensure optimal colony health.

Both mitochondrial disease and cancer have an inflammatory component. Fish and insects (e.g. flies) lack the same broad range of immune cells found in humans, which means that there are differences between fish and mammals that could affect the disease biology. Some genes are not conserved between these species and mammals therefore some of the disease mechanisms may not be the same. Therefore, drugs that target those cells or disease pathways may not work in these systems due to fundamental differences in the biology of the species compared to humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All animals, regardless of disease model are checked regularly and supportive care is readily provided to minimise distress or suffering and improve animal welfare.

We have also actively engaged in a program of low-stress handling which has shown that tube handling can reduce stress in experimental models. We have used this method in our studies for some time now and have adopted it as standard practice.

We have developed new methodologies (colonoscopy and fluorescent labelling of tumour cells for intra-vital imaging) with our collaborators and which means that we can image the tumours non- invasively over time. This means that we can more accurately measure tumour size and cull the mice at the correct point to minimise suffering.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

For all models and optional procedures good practice guides will be used to help refine the model as described in the ARRIVE guidelines, Laboratory animals special article 2015, 49 (s1) and the NC3Rs guidelines.

I am a member of the NC3Rs Oncology Network and will continually review guidance and good practice guides developed as part of this network when performing cancer studies. In addition we will implement the guidelines published by Workman et al in 2010. (British Journal of Cancer (2010) 102, 1555–1577) regarding internal cancer models to perform and monitor cancer studies.

We will also regularly consult the following sources to ensure the highest standards of animal welfare using the most refined approaches:
- The UK Home Office
- The European Union
- Code of Practice for Housing and Care of Animals Bred, Supplied or used for scientific purposes
- RSPCA Animals in Science guidelines
- UFAW Guidelines and Publications

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

There are many sources in which provide information regarding 3Rs advances, these include; the NC3Rs website, NC3Rs seminars/events and emails, scientific publications and published guidelines as well as continued professional development e.g. local seminars, regular communication with the NACWO and veterinary team and academic collaboration with the welfare group.
As information on welfare or technical improvements, alternative less severe models or new non- animal model systems becomes available an appropriate strategy within the research group and veterinary teams will be implemented to ensure that animal use and suffering is minimised. This will include testing new models (animal or non-animal) and modifying procedures.

99. Validation of chromatin regulators of cancer immunity

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

cancer, chromatin, immune checkpoints, epigenetics, anti-tumour immunity

Animal types	Life stages
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project will validate the role of chromatin regulators of gene expression in cancer clearance by the immune system. We will use a tumour cell line that grows in mice when you inject it under the skin to measure tumour growth.
Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

It is necessary to validate the efficacy of treatments designed to promote anti-tumour immunity at the whole-organism level, where we can assess tumour elimination by the innate and adaptive arms of the immune system.

What outputs do you think you will see at the end of this project?

The output of this study is that it will find new drug targets for cancer patients. Such drugs would cause a tumour to become visible by the immune system so that it can be recognized and eliminated. New treatments that harness the immune system to kill cancer are in great need particularly for lung cancer. These should be less harmful to patients than chemotherapy and radiotherapy. The findings of this work will be communicated in presentations at conferences and in publications.

Who or what will benefit from these outputs, and how?

The communication of these knowledge outputs will be during and at the end of the project as and when information is obtained. The short-term impact of this pre-clinical validation work will be that researchers/companies will build on these findings in terms of developing drug screens to find small- molecule inhibitors of these chromatin targets. Inhibition of these chromatin targets leads to expression of novel tumour-associated antigens, which can be recognized as foreign by the immune system. The T cell receptors (TCRs) and antibodies that recognize these antigens (referred to as dark antigens because they are uncharacterized) will be of interest to researchers and companies and may form the basis for therapeutic vaccines.

In the long-term, cancer patients will benefit from these studies through the development of personalised medicines. This will involve therapeutic vaccines (TCR-transgenic T cells or antibodies targeted to dark antigens), preventative vaccines and epigenetic drugs.

How will you look to maximise the outputs of this work?

We will communicate, publish and disseminate all outputs of this work. We have built a network of collaborators for this work to help us to maximise the outputs. Through our collaborators, there is a clear pathway to translation of our outputs, through startup ventures focusing on personalized medicines for cancer.

Species and numbers of animals expected to be used

- Mice:

Estimated mice: Up to 2000 C57BL/6J wildtype mice, and up to 1000 genetically-altered mice (IFNAR knockout mice, RAG1 knockout mice, CD8 knockout mice and CD19 knockout mice).

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult mice (8-12 weeks old) with a normal immune system have been chosen for this work because it represents an animal model to mirror immune responses to cancer in adults (people with non-small cell lung cancer). The strain of mice we are using is C57BL/6

because these type of mice are readily available and we will employ both male and female mice (1000 normal mice in total, which are referred to as wildtype), to reflect the fact that we are interested in immune responses to cancer in both women and men. Additionally, we will include up to 1000 mice that are genetically-modified to make them have a defect in specific parts of their immune system, which will allow us to assess which functions of the immune system are important to eliminate cancers.

Typically, what will be done to an animal used in your project?

In the standard protocol, mice will be injected under the skin in each flank with tumour cells that have been premixed with supportive transport agents like microgels. Mice will be monitored daily for their well-being and the total tumour volume will be measured three times weekly using calipers and the area of growth calculated. Mice will also be weighed regularly along with welfare clinical assessments. Mice will be sacrificed before the total tumour size (the combined tumour size on both flanks) reaches 1250mm3, which is our defined humane endpoint. After mice are sacrificed, tumours and secondary lymphoid tissues will be collected for downstream analyses. We have two additional optional steps in the protocol, one where mice will receive therapeutic immune checkpoint blockade antibodies to improve cancer clearance and one where the mice will be pre-vaccinated, which we hypothesize to improve cancer clearance. In experiments where immune checkpoint blockade antibodies are used, mice will be injected first with the tumour cells subcutaneously as described above and monitored.

Mice will then be injected twice-weekly intraperitoneally with antibodies that block immune checkpoint receptors. Well-being and tumour size will continue to be monitored regularly until the humane endpoint. In experiments where mice are pre-vaccinated, the first procedure will be to immunise the mice by injecting them intravenously with a vaccine (lentiviral vector-vaccine or mRNA vaccine). Two doses of vaccine will be given three weeks apart and one week later the mice will receive the tumour injected subcutaneously as described above. They will then be monitored for well-being and tumour size as above and culled through a schedule 1 method before the humane endpoint.

What are the expected impacts and/or adverse effects for the animals during your project?

The harm predicted in this protocol is that the mice will have tumours growing under the skin on both flanks, which may affect daily activities of the mice. The tumours may cause local inflammation (due to the injection and due to immune cell infiltration) that may cause discomfort.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Protocols have been carefully considered and tested to ensure that they are within mild to moderate levels.
C57BL/6J wildtype category: In mice that receive the unmodified tumour cell line we expect tumours to grow progressively in all mice (6-8 per group) with mice culled before they reach the humane endpoint (before the maximum combined tumour size of both left

and right-flank tumours is reached, which is 1250mm3). Mice will be monitored 3 times a week until tumours are visible and then daily for any signs of distress including visible changes (piloerection, hunched posture, decreased appetite and movement) and behavioural changes (aggressiveness). In contrast, we expect that tumours will grow less in mice that receive the gene-modified tumour cell line. Therefore, the severity is expected to be moderate in the control groups and mild to moderate in the treatment group. Genetically-modified mice (IFNAR knockout or RAG1 knockout) will be bought in for the specific experiments in which we need them: In these immunocompromised mice, we expect that tumours will grow progressively in all mice (6-8 per group) regardless of the treatment group with mice culled before the humane endpoint (before the maximum combined tumour size of both left and right-flank tumours is reached, which is 1250mm3). Therefore, the severity is expected to be moderate.

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In this specific project we need to use live animals because we are validating tumour elimination by the immune system, which is a whole organism assessment. We can't use humans/human data for this work because we are performing gene knockouts of chromatin regulators in the tumour and assessing the impact on in vivo innate and adaptive immune responses. The work proposed here will also be supported by in vitro gene knockouts in human tumours. The animal work will provide us with genetic evidence to validate these genes as controllers of anti-tumour immunity in vivo. These validated proteins will then be used to identify small molecule inhibitors in drug screens as one class of potential new cancer therapies. The animal work may also pave the way towards therapeutic vaccines by validating immune responses to tumour associated antigens that are conserved or similar in humans.

Which non-animal alternatives did you consider for use in this project? We considered human lung organoid models and patient-derived xenografts. Why were they not suitable? The human lung organoids and patient-derived xenografts (PDX) (the latter which are transplanted into immunodeficient mice) can not recapitulate all the cells of the immune system and their coordinated tumour clearance. Since this is a new project, at this time it is not known exactly which cells of the immune system will infiltrate tumours and which will be critical for tumour clearance. Our preliminary data suggest that both innate and adaptive arms of the immune system will be important in cancer clearance and these can only be assessed at the whole organism level using syngeneic tumour models where mice will have fully intact immune systems.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated number of animals is based on the minimum number per treatment group of 6-8 mice. From following the 'Reduction' strategy of the 3Rs, we reduced numbers of mice in pilot experiments to 6 per group, made possible because we are taking measurements per mouse and implanting tumours into both left and right flanks of each mouse. Most experiments will be conducted in wildtype mice using tumour cells with different gene knockouts. Key experiments will be repeated in the above genetically modified mice alongside control litter mates to determine the requirement for innate and adaptive arms of the immune system in tumour clearance.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To support reduction, we have considered the minimum number of mice required per experiment. This we have reduced to 6 mice per group, which is possible because a) we will generate pure populations of gene-knockout tumour cells before injection to limit the variability between mice, b) we will take measurements per mouse and c) we will inject tumours into both flanks to have more data points. We have already done preliminary experiments to validate that there is a significant difference in tumour growth between wildtype and our gene modified tumour cells using the the above approach and numbers of mice. Additionally, both sexes of mice will be used to avoid wastage and randomised.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have opted to purchase mice in at the time of each experiment to avoid wastage during breeding. We are also permitted to breed small numbers of GA mice on site to keep experiments economical. As well as collecting tumours, we will also collect lymph nodes, serum and spleens from every mouse to freeze so that we can go back to these to obtain more information when needed rather than setting up new experiments. Additionally, depending on the results that we obtain over time, we will seek to optimize experiments further, including reducing mouse numbers or experimental groups by applying the 3Rs.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The model that we will use is a tumour that grows well and is invisible to the immune system (non- immunogenic) in its unmodified state (Boumelha j et al. Cancer Res. 2022; Ng k et al. Nature 2023). This is a suitable model in which to validate our hypothesis that inactivation of particular chromatin regulators makes the tumour now visible to the immune system so that it is killed. It also grows uniformly and quickly so that experiments are short-term (3 weeks) with a well-defined humane endpoint (the total volume of both left and right tumours cannot reach 1250mm3). The method that we are using is to implant cells (a tumour derived from the lung) under the skin on both flanks and to measure tumour growth and monitor the mice. We can then collect the tumours and measure the tumour immune microenvironment and other parameters.

Why can't you use animals that are less sentient?

We need to use animals that have a broadly similar immune system to humans. We opted for mice because they are less sentient that other species such as primates and pigs yet still reproduce our preliminary findings obtained in humans: These key findings are that we have identified chromatin regulators that suppress retrotransposons and interferon responses in both mouse and human cells.

Therefore, although the exact loci that are regulated may be different between mice and humans (both of which we will characterize in this Programme grant), the broad concepts and mechanisms will be conserved. We need to use adult mice (8-12 weeks old) to reflect the fact that we are modeling immune responses targeting tumours that occur in adults (people with non-small cell lung cancers). We need to use live (not anaesthetised) animals that have an intact immune system. We are mainly interested in lymphocyte responses, and lymphocytes are abundant in mouse spleen, lymph nodes and blood.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will handle the mice regularly before any procedure is carried out on them and allow them time to acclimatise to the animal house before experiments. The main procedure is the injection of tumour cells under the skin and the intraperitoneal injection of antibodies that block immune checkpoint receptors. Mice will be taken to a procedure room and injections performed in an enriched environment to distract them. Trained and competent animal staff and PIL holder will carry out the injections to ensure best practice. For procedures, mice will be picked up from their cage using plastic tubes rather than picking mice up directly by their tails. Mice will be monitored daily by the PIL holder to verify that there are no signs of distress. If any mice show signs of distress, they will be monitored closely and if necessary, killed according to the humane endpoints set up on this licence.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the best practice guidelines and recommendations including all up-to-dates provided by the NC3Rs as guidance to planning, conducting and refining our animal experiments. We will also use the PREPARE guidelines for the planning and execution of

nest-refined experimental procedure (to foster normal nest-building and burrowing activities of the mice) and the ARRIVE guidelines for the reporting of animal research results. Additionally, to have more accurate and faster 3D measurements of SC tumour mass, the animal staff at Barts Cancer Institute are currently testing other devices also drawing from advice from this recent article: https://www.nature.com/articles/s41596-024-00998-w and we will follow their guidance to ensure our experiments are conducted in the most refined way.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will keep the 3Rs in mind during the duration of the project licence. For example, we will keep up to date with 'Replacement' advances in the lung organoid field so that we can assess if and when to replace parts of our in vivo work. We will extract as much data as we can per mouse from our experiments and use the results to potentially reduce the number of treatment groups of mice we need going forwards. We will also refine the length of the tumour experiments if we can and look for ways to reduce any discomfort the mice incur from the tumour growth experiments and from the initial injections. We will also apply any potential new refinements on how to better enrich the animals housing environment. We will stay informed through attending workshops and training courses and through liaising regularly with the Named Animal Care and Welfare Officer (NAWCO), Named Information Officer (NIO) and Named Veterinary Surgeon (NVS) at our Institutions.

100. Developmental and circuit mechanisms underlying the emergence of behaviours

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

mouse, brain function, brain development, behaviour, neurodevelopmental conditions

Animal types	Life stages
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand how simple forms of behaviour come about during early stages of brain development, and how changes to normal development can lead to abnormal behaviours.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

It is well known that disrupting early stages of brain developments can lead to long-term abnormal behaviours, such as those seen in neurodevelopmental disorders including autism spectrum conditions. Our current understanding of how normal behaviours are formed while the brain develops, and how disturbing brain development leads to abnormal behaviours is very limited. This work will shed light on these crucial aspects and, in the

long-term, will provide us with valuable information that will help to design rational therapies for certain neurodevelopmental conditions.

What outputs do you think you will see at the end of this project?

The main outputs of this research will be the generation of new knowledge, data and reagents. We will disseminate the findings of our research to the academic community in the form of publications, seminars and conferences. We will also present our results to the broader public through specific outreach and public engagement activities. We will deposit the data that we generate on open-access data repositories, where it will be permanently available. Any reagents that are generated will be made available to other researchers either directly or through non-profit repositories.

Who or what will benefit from these outputs, and how?

In the mid-term (3 to 5 years), the outputs of our research programme will primarily benefit other researchers working in basic and translational neuroscience. In the long-term (>5 years), the insight obtained from this research could offer prospects for developing rational therapies for neurodevelopmental conditions.

How will you look to maximise the outputs of this work?

We will maximise the outputs of this work by disseminating our findings to the academic community in the form of publications, seminars and conferences. We will also present our results to the broader public through specific outreach and public engagement activities. We will deposit the data that we generate on open-access data repositories. We will share any reagents that we generate with other researchers either directly, or through non-profit repositories.

Species and numbers of animals expected to be used

- Mice: 10000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice represent the best possible approach to studying how normal and abnormal behaviours emerge during brain development. Firstly, the brains of mice are complex enough and are comparable to the human brain. Second, mice can display sophisticated behaviours that are relevant in the context of neurodevelopmental disorders. Third, mice are easy to maintain, breed quickly and are amenable to genetic modifications. Finally, the genes whose mutations are linked to neurodevelopmental conditions are also present in mice, where they can be modified to study the disorder. In carrying out the research programme, we will use neonatal, juvenile and adult mice.

Typically, what will be done to an animal used in your project?

In some cases, neonatal, juvenile or adult mice will undergo a small surgical procedure which involves injecting viruses into specific parts of their brains. These viruses are safe, non-toxic and are specifically designed to ensure that nerve cells can produce certain molecules. In most cases, neonatal, juvenile or adult mice will undergo a surgical procedure which involves fixing a small light-weight metal bar on their skull. This allows us to head-restrain the mouse, so that its brain activity can be measured. In some cases, adult mice will undergo food or water restriction (but not both) and food or water will be provided as a reward when these mice perform certain behavioural tasks under head-restraint.

What are the expected impacts and/or adverse effects for the animals during your project?

There is a small chance (<5%) of infection occurring in the surgery site. This is indicated by redness, swelling, fluid discharge, intolerance to touch or detachment of the metal bar. On rare occassions (<5%), adverse effects of surgery may include weight loss of 10 to 15% of body weight, a ruffled coat and a slightly hunched posture. These effects will not last more than a couple of days.

Some behavioural experiments involving adult mice will require restricting either food or water intake. These experiments involve animals learning a behavioural task. In these experiments, the mice will be maintained at a target weight which is approximately 15 to 20% lower than their normal weight. Food or water will be provided as a reward upon completing the task. Adverse effects may occur in a small fraction (<10%) of these animals. These include loss of more than 25% of normal body weight, a dull and unkempt coat, lack of movement, hunched posture, repeated vocalisation and sunken eyes. In such cases, the experiment will be terminated and the animal will be euthanised.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most animals (~80%) will experience moderate severity. The remaining (~20%) will experience mild severity.

What will happen to animals used in this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our goal is to understand how behaviours emerge during brain development and how changes to normal development can lead to abnormal behaviours. Mice represent the best possible approach to studying this problem. Firstly, the brains of mice are complex enough and are comparable to the human brain. Second, mice can display sophisticated behaviours that are relevant in the context of neurodevelopmental disorders. Third, mice are easy to maintain, breed quickly and are amenable to genetic modifications. Finally, the genes whose mutations are linked to neurodevelopmental conditions are also present in mice, where they can be genetically modified to study the disorder.

Which non-animal alternatives did you consider for use in this project?

I used the NC3Rs resource library to identify suitable non-animal alternatives. These included computer modelling and simulation, organoids and *in-vitro* cell cultures.

Why were they not suitable?

Recent developments allow us to study the functioning of individual nerve cells, in conditions under which these cells are grown and kept alive outside the brain. These include organoids and *in-vitro* cell cultures. Similarly, computer modelling and simulations (*in-silico* strategies) are useful for understanding the functioning of individual nerve cells. However, to understand the emergence of normal and abnormal behaviours during brain development, it becomes necessary to study the brain as a whole in animals that can display complex behaviours. For these reasons, we need to use intact animals. In carrying out this research, we will regularly review the NC3Rs guidelines and continue to search for viable non-animal alternatives

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The experimental protocols proposed in this project are similar to those that the principal applicant has performed over the past 10 years. The estimated number of animals that each protocol will require was reached after considering previous experience, the projected number of researchers in the laboratory, pilot data, effect sizes, biological variability and attrition rates.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

During the design phase, the NC3R's Experimental Design Assistant was utilised to reduce the number of animals. For some experiments, we already have a good indication of the effect size and biological variability through pilot experiments. In these cases, a

statistical method known as "power analysis" was used. This method gives accurate estimates of the minimum number of animals that would be required to achieve meaningful results. In other cases, where the effect size and biological variability are not known, a different approach involving Mead's resource equation was used to estimate the number of animals.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In this project, we will breed different genetically modified mice to obtain offsprings with specific genotypes. The efficient management of mouse colonies is of critical importance to optimise the number of required animals. I will actively instruct new members of my laboratory in mouse handling, colony management and efficient breeding strategies. Every month, I will review the usage of animals by each lab member and advise on breeding strategies. We will maintain a digital record of every experimental animal in an electronic lab notebook (ELN).
Some experiments will generate brain tissue, which will be registered on the ELN and safely stored at low temperatures for use in future studies. We will also share this tissue with other laboratories and researchers upon request.
Before beginning a study, we will perform a small pilot experiment which will inform us regarding the likely outcomes. We will use this information to further optimise our experimental approach and animal numbers. In our experiments, we will use animals of both sexes in an unbiased manner. Mice will randomly be assigned to control or experimental groups, after controlling for age, body-weight and litter. The experimenter will be blind to the animal's identity to prevent unconcious biases. To avoid selection bias, data analysis will also be blinded i.e. the analyst will not know the experimental history or identity of the mouse being analysed. In carrying out this project, we will continue to seek statistical advice to further improve our experimental designs and reduce animal numbers.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

During this project, we will use wild-type and genetically altered (GA) mice. The mouse is the best possible animal model for this project since their brain structure and complexity is comparable to humans, and their genome can be easily manipulated. Our experiments and procedures are designed to cause the least amount of pain, suffering or distress with no lasting harm. New lab members will be trained and supervised during surgeries either by the principal investigator, or by senior experienced colleagues until they are fully competent. Strong emphasis will be placed on aseptic surgical techniques. Painkillers and antibiotics will be provided before and after surgery to quickly relieve pain and discomfort,

and to minimise the risk of infections. We will constantly aim to reduce the duration of surgeries at all stages. Finally, appropriate handling and habituation will be carried out, before beginning an experiment, to reduce stress.

The principal investigator has over 10 years of experience in carrying out the procedures detailed in this project. In carrying out this research, our group will regularly review the NC3Rs, LASA and ARRIVE guidelines, and continue to refine our experimental procedures to further minimise welfare costs. Finally, where appropriate, we will consult with the NVS and NACWO for further advice.

Why can't you use animals that are less sentient?

Our goal is to understand how behaviours emerge during brain development and how changes to normal development, such as those seen in neurodevelopmental conditions, can lead to abnormal behaviours. This requires us to study a biological system that is complex enough to be comparable to the human brain without going to higher species such as non-human primates. Less sentient species such as invertebrates are not suitable for this purpose since their brain structures are vastly simpler and cannot be compared to the vertebrate brain (including humans). For these reasons, the mouse represents the best possible animal model. Under terminal anaesthesia, brain activity is vastly different compared to the normal waking state and no readout of behaviour is possible. Therefore, these studies need to be carried out in awake behaving mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will take several precautions to minimise welfare costs (harms) for the animals. Mice will be housed in large clean cages where they will have free access to food and water. Their cages will contain play tunnels and running wheels which will help to minimise stress and improve overall general health. Strong emphasis will be placed on aseptic surgical techniques. Painkillers (analgesics) and antibiotics will be provided before and after surgery to quickly relieve pain and discomfort, and to minimise the risk of infections. After surgery, animals will be moved to a warm environment which promotes quick recovery.
Our experiments largely involve head-restraining mice to measure brain activity. To minimise discomfort and stress during experiments, mice will undergo regular handling and habituation sessions where they will learn to sit comfortably under head restraint. Habituation sessions will initially last for 10 minutes each day and the duration of restraint will gradually be increased until the animals learn to sit comfortably for at least 1 hour. The animals will be rewarded with soya milk during or at the end of each session.
To identify adverse effects, we will continuously monitor the overall health of the animal using the "Body condition score" and the "Mouse grimace scale". The experiment will be terminated and the animal will be euthanised if **(i)** the body weight at any time falls under 25% of pre-surgery/free-feeding weight or **(ii)** an infection occurs at the surgery site or **(iii)** the animal shows signs of distress such as lack of movement, hunched posture, dull coat or sunken eyes. Where appropriate, we will take further advice from the NVS and NACWO.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

In carrying out our experiments in the most refined way possible, we will be guided by the following guidelines:

1. LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery.
2. ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments).
3. Refinements to rodent head fixation and fluid/food control for neuroscience (https://doi.org/10.1016/j.jneumeth.2022.109705).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly monitor the NC3Rs website (https://nc3rs.org.uk/) and the ARRIVE guidelines website (https://arriveguidelines.org/) regarding advances in 3Rs policies and transparent reporting of animal research. We will regularly use the resources available on the NC3Rs and ARRIVE websites while designing our study and experimental procedures.

101. Mechanisms of inflammation resolution and tissue repair under metabolic stress

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Metabolic diseases, Inflammation, Atherosclerosis, Obesity, Dead cell clearance

Animal types	Life stages
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Dead cells must be quickly removed from tissues to prevent inflammation and maintain normal organ function, but this process is compromised in metabolic and inflammatory conditions. This project aims to understand what leads to this impairment and how to restore normal organ function.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Metabolic disorders, such as obesity and atherosclerosis, are responsible for over a third of all deaths worldwide. Researching the inflammation that underlies these conditions can

help us create new treatments. Since inflammation is a common factor in many metabolic diseases, targeting it could lead to promising treatment options.
One key aspect of managing inflammation is the body's ability to quickly and effectively clear away dying cells, DNA, and debris. In many diseases, this clearance process is not working properly, which makes the condition worse. Understanding these basic mechanisms is crucial for developing new treatments.
By learning how inflammation and problems with resolving it contribute to diseases like obesity and atherosclerosis, we can find new drug targets and create better treatments to prevent or lessen these diseases.

What outputs do you think you will see at the end of this project?

Outputs from this work will include generation of new knowledge, publications, and new methodologies. The findings will be shared through open-access publications, presentations in national and international meetings, and where appropriate to the lay public through newsletters and news articles.

Who or what will benefit from these outputs, and how?

In the short-term, new information obtained from this project will be useful for understanding disease mechanisms which will impact the work of other researchers in the broad area of metabolic and chronic inflammatory diseases. In the medium and long term, these findings may lead to development of new drugs and possibly impact public health policy decisions. In summary, the output of this project could impact multiple stakeholders including academic researchers, pharmaceutical industry, and policymakers.

How will you look to maximise the outputs of this work?

The project outputs will be widely disseminated by a combination of communication methodologies including peer-reviewed publications, conference presentations, lay articles in popular media, social media channels, and through public engagement.

Species and numbers of animals expected to be used

- Mice: 3400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

For this study, we will use both young and adult mice because they are great models for studying inflammation and metabolic diseases like obesity and atherosclerosis. Mice are genetically similar to humans, which makes them useful for studying complex diseases that involve a mix of genetic, environmental, and lifestyle factors—things that are hard to fully replicate in a lab dish.
Mice have a lot in common with humans when it comes to how their bodies work. They have similar immune cells, metabolic processes, and reactions to changes in diet.

Because of these similarities, we can modify mice to develop human-like diseases, giving us valuable insights into how these diseases work and how they might be treated.
Unlike lab experiments done outside of a living organism (in vitro), using mice allows to explore how diseases develop and how inflammation affects the whole body, including interactions between different organs and tissues. Most of our research will be done on adult mice, but we will use young mice in specific studies to look at how early life dietary changes can lead to obesity and affect the development of diseases over time.

Typically, what will be done to an animal used in your project?

In this study, animals will be administered substances by mouth or through injections using common methods, such as into a vein, under the skin, or into the abdomen.
To study conditions like obesity and atherosclerosis, some animals will be fed special diets, such as a high-fat diet. During the study, blood samples will be taken, which may cause brief discomfort.
At the end of the study, the animals will be put under deep anaesthesia or humanely euthanised following standard procedures.
The duration of the study depends on the condition being modelled. For instance, models of short-term inflammation, like peritonitis, usually last 1 to 7 days, while long-term conditions like obesity, atherosclerosis, and intestinal inflammation can last between 3 to 6 months.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice may feel brief pain from injections. Changes in their diet could lead to obesity and itchy skin, and obese mice may become less active. Some mice that are exposed to radiation might experience diarrhoea, dehydration, and weight loss, but this usually lasts for less than two weeks. During this time, they will be given special diets to help them recover more quickly.
Mice that develop sudden inflammation, such as peritonitis, might suffer from acute pain, decreased activity, and a mild fever. Mice with intestinal inflammation may develop diarrhoea, dehydration, and weight loss. All treatments will use the smallest effective doses and will be given by fully trained staff. The animals will be carefully monitored during and after treatment so that any side effects can be quickly identified and treated.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We do not expect any of the protocols to exceed moderate severity.

What will happen to animals used in this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Metabolic disorders and inflammatory conditions are complex and multifaceted, involving interactions between multiple cell types and organs. Animal models allow the study of these diseases in the context of a whole organism, capturing the systemic effects and interactions between different cells, organs, and tissues that cannot be replicated in vitro. Secondly, animals provide an opportunity to investigate disease progression over time, which is crucial for understanding the underlying mechanisms and identifying potential therapeutic targets.

Which non-animal alternatives did you consider for use in this project?

In vitro methodologies such as cellular coculture, 3D-cultures, and organ-on-chip were considered as non-animal alternatives.

Why were they not suitable?

In our lab, we often use co-culture systems, where different types of cells are grown together, to study how cells interact with each other. However, these systems are quite simple and don't fully capture the complexity of tissues like blood vessels and fat.
We also considered using organ-on-a-chip technology, which tries to mimic how organs work, but these models aren't advanced enough yet to replace animal research. For example, the current models of blood vessels on a chip don't have all the layers or different types of cells found in real blood vessels. Because of these limitations, organ-on-a-chip technology can't yet be used to fully understand human biology and disease.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These numbers are based on our previous usage of animals both for breeding and experimental purposes.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will calculate the minimum number of animals needed for our experiments to ensure our results are statistically meaningful while also minimising animal use. These calculations will be based on data from our previous studies. Generally, for studies on advanced atherosclerosis, we need 13-15 mice per group to detect a 30% difference with 80% confidence and a 5% error margin. For studies on acute inflammation, we expect to

need 7-8 mice per group. If we lack prior information, we will conduct small pilot studies to understand baseline variability and determine the appropriate number of animals needed for reliable results, consulting with trained statisticians. All team members will be trained in proper experimental design using available online tools like https://eda.nc3rs.org.uk.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We aim to optimize breeding strategies to ensure timely availability of sufficient numbers of genetically altered (GA) and control animals. We will manage our colonies efficiently by following guidelines from resources like the NC3Rs and taking advice from NACWO and NVS. We will incorporate imaging modalities into our experimental design to serially monitor animals for disease progression which will reduce the numbers of animals used in the study. Also, multiple organs isolated from the study will be stored appropriately for various analysis which will be readily shared with other researchers with similar interests.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The aims of the PPL are to elucidate fundamental mechanisms in dead cell clearance, acute inflammation, and chronic inflammatory pathologies. In this context, we will use animal models that best mimic pathological states and human disease processes. This will ensure the relevance of our findings to human conditions. Additionally, our choice of diet-induced atherosclerosis avoids more severe and aggressive surgical models of atherosclerosis.

Why can’t you use animals that are less sentient?

While less sentient species such as Zebrafish can provide insights into fundamental principles of innate immune cell function in the context of inflammation, they do not fully recapitulate the complexity of the inflammatory response within the vascular wall and neither do they develop advanced atherosclerosis mimicking the human condition. Moreover, the availability of high-quality and validated scientific and technical resources for mouse research enables the conduct of impactful research with applications to human disease mechanisms and the development of new therapies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animal welfare is a central consideration in all our protocols. Guided by our NACWOs and NVS, we ensure adherence to best practices and the most refined techniques. All

personnel involved in animal experiments will review literature on animal welfare recommendations provided by the local Animal Welfare and Ethical Review Body (AWERB).
Wherever possible, analgesics will be used to minimise pain and suffering. We will use disease severity scores to closely monitor animal welfare, with clearly defined humane endpoints. These scoring systems consider the animal's condition and behavior, as well as clinical severity, allowing for comprehensive monitoring. Measures to reduce suffering, such as providing supportive food and enriched housing will be implemented. In addition to following the guidance outlined in our scoring systems, we will always seek advice from the NVS or NACWO if animals reach early endpoints.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the best practice guidelines published by the RSPCA on general mouse housing and care (Mice: Good practice for housing and care, 2nd edition, April 2011) and the 3Hs Initiative (https://www.3hs-initiative.co.uk). We will refer to the PREPARE guidelines checklist for conducting high quality studies (https://norecopa.no/media/7893/prepare_checklist_english.pdf). Additionally, we will follow recommendations of Hawkins et al (RSPCA expert working group) for refining bone marrow ablation and reconstitution in mice.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will subscribe to NC3R newsletters and attend meetings and workshops to stay up to date with the latest developments.

102. Mining the microproteome as a source of novel rejuvenation targets or therapies

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

ageing, rejuvenation, microproteins, reprogramming, integrated stress response

Animal types	Life stages
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult, Aged animal

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The primary objective of this project licence is to identify new microproteins associated with the processes of ageing and rejuvenation and to decipher their potential role in ageing related diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Undertaking this work is crucial due to the significant human and economic burdens associated with ageing and its related diseases. Despite extensive research, the biological underpinnings of ageing remain poorly understood. By exploring the initial cellular and organismal events that drive ageing, this project aims to uncover fundamental insights that could lead to novel strategies for delaying, reversing, or even preventing the diseases associated with ageing.

The use of well-defined animal models in this research is pivotal for advancing our understanding of ageing mechanisms and could potentially lead to groundbreaking therapeutic approaches for agerelated diseases. This not only addresses a critical gap in scientific knowledge but also has the potential to significantly reduce the sociological and financial impacts of ageing on a global scale.

What outputs do you think you will see at the end of this project?

At the conclusion of this project, we anticipate generating several impactful outputs that will advance the field of ageing and age-related diseases. These outputs include:
Advancement in the field of ageing, potentially establishing a new sub-field focused on the concept that ageing results from a loss of resilience and can be treated to restore homeostasis. This innovative idea could reshape current research paradigms.
Identification of novel microproteins, metabolites, and bioactive products that play roles in ageing and age-related diseases, paving the way for new therapeutic strategies targeting molecular mechanisms previously overlooked.
Creation of a comprehensive atlas of ageing-related microproteins, providing a valuable resource for the research community and potentially catalysing further expansion and exploration within the microproteins field.

Who or what will benefit from these outputs, and how?

The outputs from this project will benefit both the scientific community and society at large. In the short-term, scientists will gain access to new data, methodologies, and insights through publications and presentations at conferences, enhancing the collective understanding of ageing and its mechanisms. This dissemination of knowledge will also extend to the public through organised events, increasing awareness and understanding of ageing-related research. In the long-term, the profound societal impacts of this research could lead to preventative strategies that mitigate ageing and its associated diseases. By identifying and targeting the factors that control ageing, this project aims to significantly improve health-span, reducing the prevalence and impact of age-related conditions among the elderly. This dual focus on immediate academic contributions and long-term societal benefits underscores the project's comprehensive approach to tackling the challenges of ageing.

How will you look to maximise the outputs of this work?

The results from this work will be regularly shared with the wider scientific community by making our data and findings available in the appropriate databases. Moreover, the universal atlas of microproteins in ageing across organs will provide a new resource that can be used to interrogate additional scientific questions on the more global scale of the ageing process at a cellular level. Open access to this resource will prevent further and unnecessary animal research relating to ageing as the resources are already available.

Species and numbers of animals expected to be used

- Mice: 23650

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The use of mice in these projects is justified by their biological and physiological similarities to humans, particularly in the context of ageing and its associated diseases. Mice are valuable for their ability to model complex mammalian organ systems and processes such as cancer development and fibrosis, which cannot be adequately replicated in non-mammalian models or in vitro systems. Additionally, the extensive range of genetic manipulation techniques available for mice, including transgenic, knock-in, and knock-out models, allows for precise investigations into specific aspects of ageing biology. The selection of life stages, ranging from two-thirds of the gestation period to old age, is strategically chosen to cover a comprehensive spectrum of ageing, enabling a thorough exploration of the ageing process.

Typically, what will be done to an animal used in your project?

In this project, mice will undergo various procedures depending on the specific protocol. Some mice will develop tumours, either spontaneously or through the implantation of genetically modified cells. Others will be treated with doxycycline to induce in vivo reprogramming, or with potential medicines to induce fibrosis in several organs. Throughout these experiments, the mice may undergo multiple rounds of non-invasive imaging, which requires light anaesthesia and the administration of a substance to enable visualisation of tumour growth. They may also have blood withdrawn multiple times to monitor disease progression and receive newly developed drugs or agents via injections, oral gavage, or in their drinking water. At the conclusion of the study, all animals will be euthanized using a schedule 1 method.

What are the expected impacts and/or adverse effects for the animals during your project?

During the various tests, the animals may experience the following adverse effects:

- Transducing agents: When substances are given through injections or orally, the animals may feel temporary discomfort due to the process. This discomfort can happen daily or weekly and lasts for about 10-15 minutes. In rare cases (less than 2%), repeated injections can cause tissue damage. However, most of the time, the discomfort is mild and temporary.
- Organ damage: The discomfort from organ damage depends on the substance used and the organ targeted. Animals may experience localised discomforts like breathing difficulties, stomach discomfort, and skin ulcers. Other discomforts may include lethargy, hunched posture, lack of grooming, signs of pain, and weight loss up to 20% or 15% for 3 consecutive days.
- Tumour formation: This can cause the animals' distended abdomen, lead to weight loss

(cachexia), difficulty breathing, digestive issues, changes in behaviour, lethargy, and weight loss up to 20%.
• Rejuvenation (genetic): Most mice will show no effects but some mice will lose weight during this process as the colon and pancreas become less functional whilst they rejuvenate. This process usually lasts for a week and mice regain weight once completed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Up to 80% of the animals used within this project will experience a moderate level of severity. The rest will experience up to mild levels of severity

What will happen to animals used in this project?

- Killed
- Used in other projects
- Kept alive at a licensed establishment for non-regulated purposes or possible reuse

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We perform many experiments in non-animal models however the interactions of different cellular populations within a tissue are currently too complex to recapitulate in a culture dish; for this reason it is essential to carry out some experiments on animals. This is particularly true in the context of ageing and reprogramming where models of these in cultured conditions are limited.

Which non-animal alternatives did you consider for use in this project?

We actively use and/or intend to use non-animal alternatives. We attempt to use all the methods synergistically, gaining the maximum benefit from each and reinforcing the strength of our findings. These alternatives include:
cells grown in petri dishes.
Organoid cell models; where we try and force cells to grow in 3-dimensions like mini tissues, as opposed to a flat sheet of cells in a petri dish.
Tissue cultures; where we take organs and slice them into thin sections to keep in incubators. One mouse organ can be sliced many times to reduce the numbers of mice we need to answer a scientific question.
Modelling via computational approaches.

Why were they not suitable?

Each of the models described above has advantages over animal models including a reduced financial and time cost, as well as an ethical benefit. However, these models are limited in their ability to fully recapitulate tissue states and the interactions between tissues. Additionally, models that require proliferation (cell culture or organoid models) are not well suited to ageing models which usually have defects in the capacity to proliferate. Where possible we will use these systems as they can help inform the correct types of animal models and experiments. We will also do the reverse and take our research findings from animal models and try to build better non-animal models that recapitulate these settings. We strongly believe that these models can act synergistically with animal models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The lab has been maintaining mouse colonies and performing mouse experiments for the past 6 years. The estimated number of animals we will use is partly based on our usage over that time. We consult with a professional statistician where possible and the numbers of mice that form our experimental cohorts are carefully considered to make sure we can answer our scientific questions. However, a large portion of our estimated usage arises from the breeding of genetically altered mice, combining two or more different strains of mice to make complex models.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We used a combination of our own historical data and experimentally obtained to help predict sample and effect sizes where possible. In addition we used the NC3Rs experimental design tool to assist in our study design. Our current protocols are designed to enable us to obtain as much information (tissues, blood sampling) from each experimental cohort as possible, reducing the need to re-run studies to acquire additional samples.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In our research, we often use organisms that have been genetically altered to have multiple versions of a certain gene. Because of the way genes are passed down (a principle called Mendelian inheritance), we sometimes end up with extra animals that do not have the gene mix we need. We are always trying to improve our breeding methods to get the exact types of animals we need for our experiments. We are also exploring other ways to make genetic changes, like using special viruses (AAV, lentivirus) or techniques (like LNP-mRNA delivery and CRISPR). These methods let us add or change genes without having to breed animals with those changes.

We will also run smaller "pilot" studies to help us understand the real impact of what we are studying, and we'll consider factors like the sex of the animals.
Lastly, we will collect and save as many samples as we can from each experiment. This will help us build a library of tissues we can use in future research. This not only reduces the number of new experiments we need to run, but also allows us to share samples with other researchers.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are the only species that are employed in our protocols. In particular, we will use genetically engineered mouse models (GEMMs). These strains have been specifically generated to carry the genetic mutations that cause the loss of expression of our microproteins of interest or to carry a transgene that allows the expression of the reprogramming factors in vivo. In this project we will use these strains to explore the biology of ageing and ageing-associated diseases, trying to reverse it by testing the role of our microprotein candidates in combination with reprogramming as a rejuvenation strategy. Animals in these studies will be maintained under normal conditions until a specified time, at which point material will be collected and analysed as previously described. These methods are all designed to result in no more than a temporary discomfort to the mice.
Implantation of genetically modified cells (orthotopic, subcutaneous, intravenous,) will be carried out in immunocompromised (NMRI Nu/nu) and immune competent (C57Bl/6J and our own genetically engineered animals) mice.
We will minimise suffering by adhering to the best practice guidance, currently the NCRI guidelines for the welfare and use of animals in cancer research. Every protocol proposed in this licence is the most refined for the purpose and designed to cause the minimum distress and suffering to the animals.

Why can’t you use animals that are less sentient?

Using animals at an immature life stage is not appropriate for our studies due to the fact that we are looking to study ageing and ageing related diseases development. Additionally terminally anaesthetised animals cannot be used as our experiments are conducted over days to years. For the study of tissue damage, ageing and rejuvenation we require the use of mammalian organisms with fully developed organs.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Increased monitoring will occur during periods where there is an increase in the welfare cost to the animal e.g., during tissue damage, and also as an animal ages. In addition to this, husbandry based refinements will be employed where possible e.g., food on the cage's floor such as gel diet and mash for mice with limited movement.
For drug studies we will always look to use the least invasive route for drug administration, and when testing drugs or combinations that we have no previous personal experience with we will always perform initial pilot studies before embarking on larger studies.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

When planning experiments, we will refer to the PREPARE guidelines and for general guidance in our experiments we will adhere to the LASA guidelines.
For ageing studies there are a number of studies we will consider as best practice guidelines such as: "A toolbox for the longitudinal assessment of healthspan in ageing mice" (Bellantuono I. et al. Nat
Protoc. 2020), "Ageing Research Using Mouse Models" (Ackert-Bicknell CL et al. Curr. Protoc. Mouse
Biol. 2015), and "A clinical frailty index in ageing mice: comparisons with frailty index data in humans" (Whitehead et al. J. Gerontol. A Biol. Sci. Med. Sci. 2014). In addition for cancer studies we will use best practice guidelines as "Guidelines for the welfare and use of animals in cancer research"(Workman et al. Br J Cancer. 2010).
Additionally we will use NC3Rs guidelines on breeding to minimise wastage, as well as conforming to the home office "Efficient breeding of genetically altered animals assessment framework" to ensure best practice when maintaining colonies.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We currently receive regular updates from the NC3Rs newsletter and will strive to have regular discussions with the Named Persons at our facility to ensure we are kept abreast of any major updates that may impact our work. Where possible if new protocols or refinements are reported to offer superior advancements in the field of the 3Rs we will look to implement them within our own studies, unless doing so would invalidate the results of an already established project. In this scenario, once the existing project has completed, any new projects that require the same technique would implement the new protocol. Importantly, we will also attend conferences and strongly believe in proactive discussion with groups performing experiments in the same or related fields both locally and at geographically distant locations. Sharing experiences and protocols is essential for ensuring we can achieve better scientific data with fewer mice using more refined techniques, and enables us to be kept abreast of any new techniques and technologies.

103. Livestock vaccine development

Project duration

5 years 0 months

Project purpose

Basic research
Translational or applied research with one of the following aims:
(i) Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

vaccination, livestock, Immunity, parasites, zoonotic bacteria

Animal types	Life stages
Mice	Juvenile, Adult
Sheep	Juvenile, Adult, Pregnant adult
Cattle	Neonate, Juvenile, Adult
Goats	Juvenile, Adult, Pregnant adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to develop novel and effective vaccines for control of ruminant livestock pathogens (disease causing agents) which either cause disease in the animals themselves, or cause disease in humans (zoonotic pathogens). The project will focus on gastro-intestinal nematode (GIN) parasites, which cause major production losses in sheep and cattle, and the zoonotic pathogens Shiga Toxin-producing *E. coli* (STEC) and *Coxiella burnetii*, the causative agent of Q fever, for which ruminants are the major reservoir of infection.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Current control measures for the livestock infectious diseases targeted in this project are either becoming less effective (in the case of GIN parasites where drug resistant parasites are a major problem), are not effective enough for use (in the case of STEC in cattle), or have significant safety and manufacturing issues (in the case of *C. burnetii*). There is therefore an urgent need to develop alternative control measures for GIN parasites, of which vaccines hold significant promise, and also optimise current STEC and *C. burnetii* vaccines to improve their safety, effectiveness, and ease of manufacture.

What outputs do you think you will see at the end of this project?

The outputs of this project include the development and testing of new vaccines for important infectious diseases of livestock, including those that cause direct harm to sheep, cattle and goats, and those that can cause severe disease in humans. The project will also provide new information on the immune response to these infections which can be used for development of improved diagnostic tests, or to inform other disease control strategies such as genetic selection of animals with improved disease resistance. The project will also specifically test how vaccines and other treatment strategies can be used together to optimise the control of bacterial infections in cattle. It is anticipated that over 20 vaccine formulations and treatment strategies will be assessed during the project.

Who or what will benefit from these outputs, and how?

This work is in response to national and international needs, contributing to biological, veterinary and medical knowledge and is in the public interest. The development of vaccines will reduce the disease burden of our livestock species, thus improving the health and welfare of farmed livestock, and in the case of diseases carried by livestock which infect humans, improve human health. For ruminant livestock diseases which cause production losses, it will reduce the impact of these diseases on the environment by reducing greenhouse gas (GHG) emissions intensities (the quantity of GHG emmitted per unit of production such as milk and meat), thus contributing to global Net-Zero targets by either reducing the number of ruminants that are farmed while maintaining food production, or increasing production from a stable global livestock population.
Furthermore, the development of vaccines, in some cases in combination with non-chemical treatments, will reduce the reliance on chemical treatments including antimicrobial and antiparasitic drugs to control disease. This will result in reduced contamination of the environment with drugs which have a negative impact on biodiversity and which may promote the spread of antimicrobial resistance.
The timescales for these benefits to be realised depend on the outputs. Improved knowledge of the immune response to the diseases targetted will be realised in the short-term (within 1-2 years); for vaccines, benefits will typically be realised within the medium (3-5 years) to longer term (5-10 years) depending on the current stage of development of the vaccine.

How will you look to maximise the outputs of this work?

Outputs from this project will be disseminated through open-access scientific publications, including publishing data on unsuccessful vaccine trials. Publication of this 'negative' data is important as it will prevent other research groups pursuing unsuccessful approaches. We will also disseminate the results of this project to key stakeholders including

researchers, farmers, veterinarians, policy teams and industry through a variety of approaches including social media, virtual and in-person meetings, and newsletters, and will make use of existing extensive collaborative networks to maximise the effectiveness of these knowledge exchange activities. Biological samples derived from studies will be archived for future studies, including those conducted by other researchers, and data generated from the project will be depositing research data in publicy available repositories.

For vaccine development programmes, the aim is to develop commercial vaccines that will be used in the field. To this end, we are currently and will continue to actively engage with companies interested in commercialising the vaccines targetted by this project. Promising vaccines will be commercialised through appropriate licensing agreements with these companies.

Species and numbers of animals expected to be used

- Mice: 400
- Cattle: 300
- Sheep: 300
- Goats: 100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The animals used in this work are the natural hosts for the diseases being studied, with the exception of mice which will be used in some instances to refine prototype vaccines prior to testing in sheep and goats. For parasite vaccines, we will use cattle and sheep at a young age (< 1 year old) when the parasites cause most disease and therefore when the vaccines need to be most effective. Similarly for *E. coli* vaccines, we will use cattle at ages when control of this bacteria is most important (neonatal/juvenile and near slaughter). *C. burnetii* vaccines will be tested in sheep and goats during pregnancy, as infections cause abnormal pregancies and bacterial shedding around birth.

Typically, what will be done to an animal used in your project?

Sheep, cattle and goats will be housed in standard farm accomodation pens and in social groups. Mice will be housed in cages with environmental enrichment. Typically, animals will be sampled prior to the start of a study to ensure they are not infected with the disease causing organism (pathogen). Animals will then be vaccinated with a prototype vaccine through different routes including intra-muscular, subcutaneous or intra-nasal routes, typically up to three times at 2-4 weekly intervals. Following vaccination animals may be challenged with a pathogen via oral, subcutaneous, intraperitoneal or intranasal routes. Samples taken post-challenge over several weeks (usually less than 10 weeks) to determine the effectivenes of the vaccine in reducing infection. These samples will include faecal samples to dermine levels of bacterial shedding and production of parasite eggs, and vaginal and milk samples to determine bacterial shedding. Blood samples will be

collected throughout the studies to assess the immune response to the vaccines and pathogen challenge.

What are the expected impacts and/or adverse effects for the animals during your project?

The experimental models used in this work have been developed over a number of years to in order to minimise suffereing by the animals.
Blood sampling and immunisation is anticipated to cause only minimal transient irritation, such as local swelling at the injection site and a mild fever which will usually resolve in 2-3 days. Rarely, more severe persistent or recurrent swelling, abscessation or ulceration of the injection site may be observed.
Gastro-intestinal parasite challenge of sheep and cattle is not expected to cause any clinical disease, but in rare instances may cause symptoms of inappetance, gastroenteritis, weight-loss and anaemia which would require intervention if lasting more than 2 days.
Challenge of cattle with Shiga Toxin-producing *E. coli* (STEC) may cause mild transient fever and selfresolving diarrhoea of 2 days duration, although usually causes no clinical disease.
Challenge of sheep and goats with *C. burnetii* may cause a transient fever and mild swelling at the injection site of <5 days duration. Challenge may result in abortions or birth of weak offspring in 7080% of individuals, usually within 4 weeks of challenge. Animals may exhibit mild behavioural changes including isolation from pen mates and crouching/recumbency within 1-2 days prior to abortion which usually resolves within 2-3 days of birth. Challenge of mice with *C. burnetii* may cause mild weight loss and a transient fever, and swelling at the injection site of 2-3 days duration.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Blood sampling and immunisation is expected to cause mild signs in most animals, with moderate signs in < 10% of animals.
Parasite challenge of cattle and sheep is expected to cause no clinical signs or mild signs in most animals, with moderate signs in < 10% of animals.
STEC challenge of cattle is expected to cause no clinical signs or mild signs in most animals, with moderate signs in < 10% of animals.
C. burnetii challenge of sheep and goats is expected to cause mild clincal signs in most animals, with moderate signs in < 10% of animals.
C. burnetii challenge of mice is expected to cause mild clincal signs in most animals, with moderate signs in < 10% of animals.

What will happen to animals used in this project?

- Killed
- Kept alive at a licensed establishment for non-regulated purposes or possible reuse
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Wherever possible, the use of non-animal models will be employed throughout the project. However, the development of vaccines requires firstly, an understanding of the mechanisms by which an animal becomes immune to disease; secondly, which parts of the disease causing organism (the pathogen) to target through vaccination; and thirdly, the characterisation of the immune response generated following vaccination. To address these questions, we need to study the immune response as a whole (i.e., *in vivo*), as many different components of the immune system interact to generate the final immune response. Furthermore, some of the pathogens under investigation cannot survive outside the animals and we need to infect animals to obtain pathogens for subsequent infection studies and/or to provide pathogen material to identify new vaccine targets.

Which non-animal alternatives did you consider for use in this project?

This project will exploit organoid culture systems recently developed by the group which will be used to study interactions between the animal host and the pathogen. These culture are derived from stem cells from animals and replicate many aspects of the tissue of origin, incuding the three-dimensional structures and cell-types of the original tissues. Organoids can be cultured in the laboratory almost indefinitely, allow studies involving large numbers of replicates to be performed without the need to collect new animal tissues. We will also use recombinant protein technologies to generate antibodies in the laboratory which are identical to antibodies generated by the animals themselves, thus reducing the need to use animals to generate these antibodies.

Why were they not suitable?

While organoids are useful in studying some aspects of the disease process, they do not replicate more complex interactions between the pathogen and other cell types, including those of the immune system, and cannot be used to definitively assess the impact of vaccination on the pathogen (i.e., how effective a vaccine is). Therefore, while complementary, organoid studies cannot fully replace the whole animal when studing the immune response and protective effects generated by vaccination. Laboratory-generated antibodies still require sequence information from B cells (antibody producing immune cells) induced by vaccination or infection, and therefore cannot fully replace animal use.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number animals used in each protocol has been estimated from data generated in previous studies in terms of the number of animals required to obtain meaningful data, as well as the objectives of the funded research projects. We will engage with statisticians who will use our existing data, or data from other relevant studies, to calculate the minimum number of animals to be used per treatment to obtain statistically robust data. Where this data is not available, we will perform pilot studies to generate this data to subsequently calculate group sizes of subsequent studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We accessed expert statistical advice to ensure the experimental design of this project is optimal in terms of obtaining robust data. This advice also included determining randomisation methods, the most appropriate use of controls, and the statistical analysis methods required. Furthermore, we used the NC3Rs' experimental design guidance and experimental design assistant (EDA) to plan the experimental design of the proposed studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We aim to use the minimum number of animals possible to achieve the project aims by:
Using pre-existing pilot data from our work or other researchers to determine optimal animal numbers in order to minimise the number of pilot studies we need to perform.
Ensuring we use tissues harvested from other studies are used to address our research objectives rather than generating new tissues; similarly, we will create biobanks of tissues collected at postmortem for our own use and other researchers working in similar areas.
Continual review of the scientific literature to avoid duplication of other animal studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Over the last 20 years, we have developed relevant, reliable and reproducible animal disease models, which have been refined to be the least severe necessary for valid results to be obtained. Indeed, most of our disease models cause no clinical disease. Vaccination protocols to be used in this project are designed to be compatible with standard farming practices to facilitate the deployment of the developed vaccines for field use.
Considerable care an attention has gone into refining sampling techniques employed to monitor immune responses and pathogen burdens during animal studies in order to reduce the degree and duration of any suffering to a minimum. Trained and experienced

observers monitor animals at regular intervals, accurately evaluating the responses of individual animals and seeking prompt veterinary advice where necessary.

Why can’t you use animals that are less sentient?

Non-mammalian anmals are limited in their use as their immune system is different to the ruminant immune system. We cannot use embryos or very young animals due to the immaturity of the immune system at these life-stages, which in ruminants is only fully mature at around 3 months of age.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have established species and protocol specific scoring sheets to ensure accurate monitoring of the animals on study. Where possible, we will train animals through 'mock-sampling' approaches to reduce the stress associated with sampling and handling. We will also ensure animals are aclimatised for at least a week before the start of a study, are housed in stable groups to minimise stress, and are never housed alone.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will consult with PREPARE guidelines https://norecopa.no/prepare when designing our animal experiments, as well as other resources avaliable from the NC3Rs (https://www.nc3rs.org.uk/) and LASA (https://www.lasa.co.uk/) websites.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We regularly check the NC3Rs website and have an active NC3Rs committe which we interact with on at least montly basis. We receive regular e-mail updates from both NC3Rs and our internal committee, and regularly participate in Laboratory Animal Science Association (LASA) meetings. Relevant advances in the 3Rs will be incorporated into the design of our experiments.

104. Breeding Genetically Altered rodents and embryo production

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Breeding, Embryo production

Animal types	Life stages
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult
Rats	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To provide Genetically Altered rodents and wild type embryos to the research community in the establishment or external groups using our facilities.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

There are several common use Genetically Altered colonies used in various projects in our establishment and by providing these strains to the various projects we can ensure that the colonies are bred under best practice guidelines. We work within guidelines which are taken from the Genetically Altered breeding initiative from the Home Office in relation to

colonies which will be held under this service licence. We regularly review the number of rodents to ensure that the demand is met while preventing the overproduction of rodents. Some Genetically Altered colonies will be maintained for the use of tissue collection only. By maintaining the Genetically Altered colonies on the service licence we can ensure that the colonies are bred under best practice guidelines.

The similarities between mouse and human early development allow fertility studies to use mouse embryos (0.5-2.5 days post coitum) to carry out feasibility studies and develop techniques before they are applied to the human. For example, this allows to test the functional ability of synthetic proteins which has been shown to have an effect on human fertility. This type of infertility affects approximately 1200 couples per year in the UK. The mouse work is a prelude to the work translating to human oocytes.
The use of Genetically Altered embryos will only occur when we are cryopreserving Genetically Altered colonies which are held under this service licence.

What outputs do you think you will see at the end of this project?

We will have animals which are good quality in terms of genetics as we will follow best practice. This involves refreshing the background of strains by mating them out to the genetic background every 5-10 generations.
Strains with either mixed or ambiguous background strain information will undergo genetic monitoring to confirm the background substrain. By carrying out genetic monitoring it will give confidence to the reproducibility of the work.
We also use the breeding data which is generated from Genetically Altered colonies maintained under the service licence in colony sizing calculations. Using sizing calculations will help to make sure that the colonies are sized to produce the number of animals required for experiments whilst also not overproducing animals which will be culled as surplus.
Data will allow us to calculate mortality rates of individual Genetically Altered colonies, thus removing the need to refer to genetic background strain data.

Who or what will benefit from these outputs, and how?

The research community or external groups using our facilities capabilities will benefit as we will produce good quality animals for their research with accurate breeding and phenotyping data. By producing animals with good genetic integrity, the reproducibility of experiments is improved. Using colony calculations, we can reduce the number of animals being produced and potentially wasted.

How will you look to maximise the outputs of this work?

As a large establishment, there may be duplication of Genetically Altered colonies and by offering to breed and maintain the Genetically Altered colonies under one service licence we can reduce the number of mice and rats which are potentially wasted. The use of the service licence will be disseminated through the establishment welfare meetings. At the meetings, the benefits of using the licence will be discussed e.g. reducing the number of animals wasted, genetic integrity and reproducibility.
Within our regular reviews of colonies at the establishment, we can specifically target groups where we identify an issue with the breeding and promote the use of the service licence as an alternative.

Species and numbers of animals expected to be used

- Mice: 31,000
- Rats: 1,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

In order to fully understand the effects of human genes and their disease-associated mutants, there is no suitable substitute for a mammalian model for certain disease models. Due to the complex interactions which take place between different cells, tissues and organs, this can only be replicated in a living organism.
Scientists studying fertility use early-stage mouse embryos (0.5-2.5 days post coitum) before applying several techniques to human embryos. The mouse ova enable them to test the feasibility of techniques/compounds.

For cryopreservation, we will use females between the age of 3-4 weeks old as it has been shown that females in this age bracket produce a higher yield of ova and a higher percentage of females which are mated produce fertilised ova.

Typically, what will be done to an animal used in your project?

Most animals on the licence will be bred and maintained with no welfare issues. Mice and rats will be maintained to a maximum of fifteen months old. Where required animals will be earmarked to obtain a sample for genotyping. The earmarking will generally take place when the animal is around 2 weeks old.

Some female mice will receive intraperitoneal injection of hormones 46-48 hours apart to generate either wild-type embryos for groups studying fertility, or external groups or to generate transgenic embryos for the cryopreservation of lines. The hormone injections are used to increase the number of ova which is obtained from each female. By using this method, we can use fewer animals to obtain the required number of ova for scientific outputs. The females are then culled by a schedule 1 method to harvest the embryos/oocytes. The cryopreservation of lines is to either remove a non-used line from the shelf reducing wastage or to safeguard from a disease outbreak or genetic contamination. Female mice can be left unmated after the hormone injection depending on the researcher's requirement.

What are the expected impacts and/or adverse effects for the animals during your project?

There are many possible adverse effects due to genetic modification of the genome. Information on expected phenotypes will be collected before breeding and will be recorded in a colony passport. Any animals exhibiting any unexpected harmful phenotypes will be humanely killed. In the unlikely event of any animal showing any signs of suffering greater

than minor and longer than transient or that compromises normal behaviour in any way, the animal will be humanely killed.
Ear notching should involve only slight and transient pain and no healing problems.
Intraperitoneal injections should involve only slight and transient pain.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice: 62% subthreshold, 38% mild
Rats: 90% subthreshold, 10% mild

What will happen to animals used in this project?

- Used in other projects
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Mice and rats are required to supply the needs of end-users Project Licences or external groups using our facilities' capabilities. To fully understand the effects of human genes and their disease-associated mutants, there is no suitable substitute for a mammalian model. The mouse and rat are the models of choice for genetic modification modelling human diseases because of the availability and ease of manipulation of the mouse embryonic stem cells. The justification for their use lies with the end-users Project Licence and cell culture assays will be used wherever possible.
Scientists studying fertility or other external groups using our facilities capabilities require live earlystage embryos (0.5-2.5 days post coitum)

Which non-animal alternatives did you consider for use in this project?

The aim of the service licence is to provide end-users with mice and rats for their studies and the consideration of non-animal alternatives will differ depending on the end users' area of research.
Any researcher who wishes to use the service licence will need to provide justification of why they wish to use animals under the service licence and details of any non-animal alternatives they have considered.
On the request to house animals on the service licence, the end-users will need to detail which searches and search engines they have used to find non-animal alternatives.

Why were they not suitable?

The aim of the service licence is to provide end users with mice and rats for their studies and will only be done when non animal alternatives have been considered and rejected. The reasons for this will differ between projects but typically the reasons for this are that a whole organism is required for systems biology or behavioural research.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The majority of this licence concerns the breeding of mice and rats. We have extensive experience of calculating animal numbers on a previous licence and we plan to carry on with this practice. We work with end-users to calculate the minimum number required to reach their scientific goals. This involves estimating the final number of animals required and then calculating the number of breeding animals required using existing best-practice guidance. This includes ensuring the appropriate age of animals used for breeding, replacement of breeding animals before productivity declines and the use of production efficiency index calculations to size the colony. All colonies on the service licence undergo a three-weekly review where the productivity of each breeding pair/trio is assessed and any which fall outside of our guidance are removed.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The size of colonies held on this licence will initially be calculated on the need of the end-users. They will be expected to provide a rationale for the number of animals required to meet their scientific goal and we will calculate the number of breeding animals required using known breeding data.
Genetically Altered colonies held on the service licence will be subject to regular reviews of the breeding performance. The actual breeding performance will be assessed during these regular reviews using data held on a database. It is this data which is used to calculate colony sizes and identify breeding pairs/trios which fall outside of our guidance. Where usage of a line has fallen to a point where it is no longer possible to breed efficiently, for example, a line being kept on 'tick-over' and not currently being used for experimental work, we will suggest to the users that we will cryopreserve the line. This reduces the number of animals being produced unnecessarily.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The primary purpose of this service licence is the efficient breeding of Genetically Altered animals.

All strains which are maintained on the service licence will be overseen by experienced animal technicians to ensure that the breeding programmes devised for the colonies are

followed. This will help minimise any potential wastage from overbreeding. Where appropriate lines will be cryopreserved to prevent animals from being produced unnecessarily.
This service licence also provides the opportunity to consolidate the breeding of models used by several end users. This would prevent the wasteful maintenance of several smaller separate colonies to provide each of the end-users with these animals. An example of this would be commonly used cre lines (i.e. lines with site-specific genetic locations).
Animals are promptly sampled for genotyping so that any animals which are of the incorrect genotype are not kept within the colony. End users must demonstrate that the genotyping protocol is validated and robust before a newly generated model is allowed onto the licence.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use models of genetically altered rodents. This will include models relevant to neuroscience (such lines which are used as models of motor neurone disease), cardiology (such as lines with altered blood vessel function) and other areas of biomedical science (such as lines with altered calcium signalling, important in the immune response). By consolidating the breeding of these lines and monitoring with dedicated staff, we can ensure that breeding and maintenance are done in the most refined manner. This includes efficient breeding practices and the calculations used to size the colonies. We also regularly bring in new breeding stock from the same breed to keep the animals healthy and make sure important genes don't accidentally get lost over time.
Where a line is no longer being actively used, it would be cryopreserved which involves superovulation (that is, the stimulation of ova). This is performed using a very established procedure consisting of timed injections prior to breeding and is considered to be the most refined.

Why can't you use animals that are less sentient?

The aim of this project is to provide end-users with mice and rats, the justification for which will depend on their individual scientific goals. Typically, the justification for using rodents is that lower-order organisms do not exhibit the required features to accomplish their scientific aims. For example, an adaptive immune system which is absent from species such as flies. Increasingly, rats are being used for more complex cognitive tasks which mice are unable to learn, so there will be instances where endusers use rats over mice or other species. End users will be required to provide justification to use models on this service licence.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

By using efficient breeding and colony management we can reduce the number of animals being produced which are being culled as surplus. Our guidelines ensure that breeding animals are retired from breeding by 8 months of age which reduces any potential for breeding related complications.

Where substances are administered by injection, the minimum effective dose and the most refined route will be chosen.

When tissue is required for genotyping ear punch is the default position.
Whilst this service licence includes the option for producing animals for cryopreservation by embryo, we will always recommend sperm freezing as the most refined method of cryopreservation, before we agree to carry out embryo cryopreservation.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Home Office efficient breeding of Genetically Altered animals
We will use the PREPARE guidelines and guidance from NC3Rs and Laboratory Animal Science Association

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have guidelines in place for breeding colonies which ensure effective breeding of rodent colonies in line with the principles of the 3R's. These are applied to the breeding on the service licence but also distributed through the establishment to other licence holders who maintain breeding colonies. These guidelines will be regularly reviewed based on any advancements made in this area. These advancements will be identified through regular attendance of conferences (LASA, IAT Congress, NC3R's tech symposium, etc.) and comparisons to other large breeding institutions. We receive frequent updates from these large breeding institutes as well as information from the NC3R's, Norecopa and our internal 3R's subcommittee. The 3R's subcommittee collects advancements from across the establishment and disseminates this information. This enables the wider implementation of the advancements made by individual groups.