Home Office

Animals (Scientific Procedures) Act 1986

Non-technical summaries for project licences granted January – March 2025

Contents

1. Targeted immune and molecular therapies 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
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

cancer, immunotherapy, antibodies, immune cells, immune response

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 study the immune and molecular mechanisms associated with the development and spread of cancer and to discover and evaluate novel treatments. We are particularly focusing on tissue cancers such as malignant melanoma, breast and ovarian carcinomas, especially subtypes of these diseases for which there are few effective therapies available. Our studies will help us understand how our immune system, which protects us from infections, interacts with cancer cells. We will utilise this knowledge to develop therapeutic strategies can ultimately benefit patients with 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?

Despite some promising treatments available in the clinic, solid tumours residing in tissues (in contrast to blood cancers) represent a major group of diseases for which limited effective therapies exist. Although surgery, radiotherapy, and chemotherapy have been used, tissue cancers in their advanced stages are notoriously resistant to conventional drugs and therefore present a major therapeutic challenge. Novel biological therapies, including antibodies, designed to fight tissue cancers are now emerging as important clinical management tools. Immuno-therapies for cancer have been the focus of many studies because tissue tumours are known to elicit immune responses resulting in immune cell activation. Tumour cell surface markers and biochemical processes have been targeted using molecular or immune therapies. Antibody-based drugs are established in medical treatment against autoimmune diseases, transplant rejection and cancer. In cancer therapy, cancer-seeking antibodies home in on malignant cells expressing their target. This can result in cancer cell death by several mechanisms, including blocking vital cancer growth functions, by immunological mechanisms or by antibodies bringing toxic payloads directly to cancer cells. Although some antibody drugs are already approved for clinical use in oncology, many tumours are inherently resistant, and others develop resistance following treatment. Therefore, the potential for antibody-based treatment of tissue cancers is far from being realised and how these treatments kill tumour cells is not fully understood. The aim of our research is to understand the underlying mechanisms of immune responses to cancer and to use this knowledge to design, study and evaluate novel biological treatments that can help control cancer progression and metastasis and can ultimately benefit patients who suffer from cancer.

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

Expected Benefits

Dissecting the mechanisms of cancer pathogenesis and the recruitment and alterations of immune cells and their signalling in cancer will further our understanding of how tumours grow and how different components of the immune system respond to cancer and inflammatory stimuli. We aim to examine ways of modulating these responses with the view of “alerting” the immune system, to inform novel immune therapy design. We will strive to dissect the molecular mechanisms underlying tumour growth and metastatic potential. In vivo models described herein, used in combination with in vitro, ex vivo assays and tissue and tumour organoid models, will contribute to the design and translational potential of therapeutic and biomarker approaches.

Antibodies can be made in the laboratory and normally possess a single recognition element to a target antigen. Antibodies targeting a limited group of target antigens on cancer cells (tumour- associated antigens, TAAs) and checkpoint targets which can be found on immune cells, provide clear survival benefits in several cancers. There is an urgent need to build and improve upon the well- documented efficacy of antibodies such as Rituximab, Trastuzumab, or antibody-drug conjugates (ADC) such as Trastuzumab-DM1 (T-DM1) to benefit patients who can only receive palliative care. We are working towards developing novel antibodies for aggressive and incurable malignant diseases. An antibody generated by our team has already successfully completed a first-in-man clinical trial. The antibody showed a favourable safety profile and early signs of efficacy. The pre-clinical in vivo studies conducted under the Lead Investigator's previous Project Licences on this agent have been pivotal to the success of this antibody in the clinic. This agent is currently undergoing a more advanced trial in patients with solid tumours, opening the avenue for other antibodies of this class to be developed and tested in patients with late-

stage and incurable cancers. Our team and colleagues are working on several novel antibody technologies which harbour significant potential for translation to the clinic. Studies in this Project Licence will inform research on the lead antibody which is progressing in clinical testing and on other antibodies of this and other isotypes and novel antibody formats and may be pivotal in expediting and informing their path from the lab bench to the patient bedside. Research models described here are crucial to downstream evaluations for our most promising novel candidates and are required to help gain regulatory approval. If successful, our approaches are set to lead to improvements in the efficacy of a wide range of therapeutic strategies, including antibodies already in clinical use in conventional non-optimised forms.

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

We dissect the immunological and molecular mechanisms underlying tumour growth and immune surveillance and test these findings in vitro, ex vivo and in selected in vivo assays to evaluate their physiological significance. Our translational research teams are focusing on the discovery and evaluation of cancer therapeutics such as antibody, antibody-drug conjugate biological modalities, immune cell treatments and vaccination strategies. We also dissect the mechanisms of action of these interventions using a variety of in vitro, ex vivo assays, organoid models including those derived from patient tumour samples and with patient-derived immune cells. These studies have directly resulted in the progress of some of these strategies into pre-clinical development and clinical trials in patients. We are keen to continue and improve on our discovery and efficacy studies for cancer therapeutics. We anticipate that the findings, complemented with refined in vivo studies, will guide the discovery of more effective interventions. If, as our work has shown thus far, our strategies prove efficacious, our research will have a significant impact on cancer care and directly lead to more effective treatments.

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

We will strive to disseminate relevant research results to the general public in an appropriate form. The Lead Investigator is active in public engagement initiatives and has a track record of public engagement and outreach activities through and interviews addressed to lay audiences including patient support groups, and public engagement events. The Lead Investigator is also a member of a patient and public led local research panel for cancer research and is regularly invited to speak with patients and their families, at secondary school events, to the public and health and charitable organisation professional groups. Public engagement through these and similar formats will be pursued. Where appropriate, we will also promote new findings from this project to the wider community and lay public by communication and education such as by issuing press releases in conjunction with our institution’s press office, which will ensure dissemination through the institutional website and in the local and national press. Our antibody discovery, cancer immunity and antibody immunotherapy research has already featured in newsletters and press releases.

To ensure that our research will benefit the academic community, any data produced from our research will be presented through oral or poster presentations at international and national conferences. Additionally, we maintain strong collaborative links with international groups working within the fields of antibody engineering, cancer immunology and immunotherapy, sharing ideas, resources and data, all instigated and supported through different consortium initiatives, dedicated meetings, conferences and original and position paper publication projects. Interaction forums include the Antibody Society, the American

Association for Cancer Research (AACR), EuroMabNet, the European Academy of Allergy and Clinical Immunology (EAACI), The Folate Receptor Society, the Gordon Conferences and others. These forums will continue to provide important means for dissemination of scientific knowledge to enhance our understanding of cancer immunology, network, collaborate and support innovation and translation of new treatments.

Species and numbers of animals expected to be used

- Mice: 6000
- Rats: 4700

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 chosen as experimental animals as they have the lowest neurophysiological sensitivity while still having an immune system of comparable complexity to the human and are therefore the most frequently used animals in studies of human pathology/immunopathology. We will be using adult mice since the cancer types we are studying largely occur in human adults. In many of our studies, we select mice in which we can engraft human tumours since our antibody therapeutic treatments target human cancer cells. For this reason, we select mice with partly intact immune systems as this gives a better chance for the tumours to grow. There are key differences between mouse and human immune cells so some experiments will involve engrafting the mouse with human immune cells which the human antibodies engage with to restrict human cancer growth. This way, we can more closely study a human antibody engaging human immune cells against a human cancer.

Rats are important to our work involving IgE-based therapeutics since the IgE immune response in humans and rats bears many similarities. This is not the case for mice which have a different set of immune cells responding to IgE antibodies. We have developed several fully syngeneic rat models of cancer which do not require human cell engraftment. We use adult rats with fully-developed immune systems, since the cancer types we are studying largely occur in human adults. One of these rat models was approved by the MHRA as the closest model to a human system to undertake efficacy and safety testing of an IgE-based drug prior to its clinical testing.

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

Typically, animals will receive a tumour challenge followed by treatments which may include human immune cells and therapeutic interventions. Tumour growth is monitored very closely, and animals are humanely killed at the end of the experiment or when the tumours reach a certain size. Monitoring and sampling to understand the effects of treatments is conducted during the study and at the end of the study. The most invasive procedures are those of tumour transplantation into the skin (skin cancer transplant models) or the mammary fat pad (breast cancer) conducted under anaesthesia. The reason for the transplantation into the specific sites is because the skin and breast, respectively, are the anatomic locations where these cancers originate and disseminate from in patients. Therefore, these models are designed to mimic the human disease and

its anatomical location of origin. The protocols used involve least pain, suffering or distress or lasting harm for the animals. A typical experiment from tumour challenge to treatment and completion of study lasts around six weeks.

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

We will establish and study rodent models of cancer and assess the effects of various treatments on helping or preventing tumours from growing. The experimental design used will involve least pain, suffering or distress or lasting harm for the animals. Engraftment of tumours may cause some pain and distress to the animals, but animals will be euthanised in case of adverse effects such as ulceration, impediment of a vital function such as locomotion, vision, mastication, excretion, weight loss or physical distress, and in any case when measurable tumours reach a specified size.

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)?

None of the intended procedures reach beyond the moderate level of severity. Procedures reaching moderate severity arise from the induction of the disease model (cancer) and are necessary to understand the immunobiology of tumours and to evaluate the effects of potential therapies. The proportion of mice experiencing a moderate level of severity is 78%. The proportion of rats experiencing a moderate level of severity is 88%. The remainder of animals will experience mild 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?

We wish to study the immune and molecular pathways that lead to the development and dissemination of cancer and by using this knowledge to develop novel immunological, molecular and biological approaches to cancer treatment, and to evaluate these as diagnostic and therapeutic tools in appropriate disease models. The in vivo studies are essential to facilitate functional and efficacy assessments for the treatment of patients with cancer. A major aspect of our research incorporates at an early stage and throughout the lifecycle of this programme, the use of in vitro screening, selection, characterisation, functional, mechanistic and efficacy assays, as well as interrogating three- dimensional tumour organoid and patient-derived organoid models and assays. These serve to significantly replace, reduce and refine all proposed in vivo experimental systems.

Overall, the majority of our investigations are conducted using patient samples and human

volunteer- derived cells, in vitro multiplex evaluations, and ex vivo assays, and are supplemented by genomic/transcriptomic, spatial transcriptomic and immunohistochemical evaluations of human specimens. When absolutely necessary to conduct experiments in vivo, we have chosen rodents as the experimental animal of choice as they have the lowest neurophysiological sensitivity while still having an immune system of comparable complexity to humans.

Animals will be bred or purchased for each experiment, and we will endeavour to use all animals with minimal wastage. There will be no crossbreeding so all animals bred will be of the desired genotype. Animals will be humanely euthanised at the end of each experiment or according to agreed protocols to minimise suffering due to tumour growth.

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

Our successful work has led to the establishment and validation of numerous assays and model systems which replace and reduce in vivo studies and are designed to elucidate the mechanisms by which therapeutics including antibodies exert their anti-tumour effects against cancer cells. These technologies often supersede the use of animal models for evaluating cellular interactions in the tumour microenvironment. Examples of some of our validated in vitro/ex vivo models and assays are:

- Spatial transcriptomic, bulk and single-cell transcriptomic analyses to interrogate the complexity of interactions between immune cells, cancer cells and different components in the tumour microenvironment
- Cell culture-based studies using patient specimens
- In vitro tumour equivalent organotypic/organoid culture models, including patient-derived organoid models
- Cancer cell viability and functional assays to evaluate the impact of potential therapies on cancer cell functions
- Assays to evaluate the ability of antibodies to activate immune cells to kill cancer cells
- Microscopy, including live imaging and multiparameter assays to study cell functions and cell-cell interactions.

Why were they not suitable?

The starting point and the majority of our investigations on cancer pathogenesis, immune responses and therapeutics discovery and functions are conducted using patient samples, human-derived cells, ex vivo and in vitro culture and mechanistic assays. We utilise cell culture and cultured organ models as much as possible to develop and evaluate our therapeutic strategies. However, characterisation in live animals prior to clinical translation is essential and often a requirement by regulatory agencies to ensure that medicines are safe and efficacious. The hypotheses generated can only be further examined using in vivo models. Routinely, we precede in vivo studies by thorough in vitro culture system assessments. Data obtained from these will help select the most promising therapeutic candidates for in vivo experiments, thus minimising and refining these. Together, in vitro and in vivo studies will help to build a clearer picture of pathogenesis, spread and immune surveillance in cancer and help evaluate key molecular and biological modalities and cell-based interventions for further development and clinical testing. Some in vivo experiments may be conducted in parallel with cell analyses in order to maximise the information derived.

We wish to conduct a reduced and refined set of animal studies for the following reasons:

1) Due to ethical limitations of investigations in humans we depend on the use of rodents to perform significant and scientifically valid research and pre-clinical testing of therapeutics.

2) There is no adequate in vitro or ex vivo model to assess the value of targeting key pathways in tissue cancers. The complexity of the mechanisms involved in carcinogenesis and how therapeutic agents interact with and interfere with these processes at the systemic level (e.g., pharmacokinetic and pharmacodynamic attributes of antibodies and ADCs, their interactions with immune cells and other components in the circulation; recruitment of antibodies and immune cells to tumours; subsequent cross-talk with cancer cells and tissue resident and tumour resident, molecular, biological, immune and immune cell components, cells, soluble factors; and interactions between several cell types in particular microenvironments) cannot be faithfully reproduced in vitro or fully recapitulated ex vivo, necessitating a refined set of in vivo experiments.

3) Due to the biological complexity of our studies, animals must be used when measuring the effects of treatments and the consequences on remodelling and repair processes which result from changes in all the cell types within the body. The latter are in turn modulated by circulating agents such as cytokines, as well as circulating cells. These processes cannot, therefore, be modelled only in cell culture or in human organoid and organotypic culture systems.

4) Since molecular, chemotherapeutic and biological therapeutics (e.g., antibodies, ADC) may be sequestered in different parts of the body, it is necessary to study their effects, tissue sequestering, biodistribution, presence and retention in tumours, in various organs and excretory pathways, to assess likely function, efficacy, toxicity and dosimetry in advance of translation to clinical testing in patients.

5) Although animals must be used in this project, this research will not only lead to the design of more effective biological treatments for tissue cancers, but in the future, some of our methods such as those for generating antibodies from human tissues has the potential to eliminate the need to use further animals to generate antibodies for the treatment of many diseases, including cancer.

6) For any targeted therapies to progress to clinical development, major insights into their pre-clinical efficacy for cancer therapy can only be derived from the use of relevant animal models of local and metastatic cancers.

Therefore, while we endeavour to utilise cell culture and cultured human organoid models as much as possible in evaluating therapeutics, their characterisation in live animals prior to translation to the clinic is essential and often a requirement by regulatory agencies responsible for ensuring that medicines work and are acceptably safe and efficacious. Therefore, in vivo studies are a necessary step in translational research pathways, to afford a prediction of therapeutic potential before clinical trials in patients for whom limited therapeutic options are available.

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 in vivo experiments are planned based on our investigations conducted using patient samples and human volunteer-derived cells, in vitro multiplex evaluations, and ex vivo assays, and are supplemented by genomic/transcriptomic, spatial transcriptomic and immunohistochemical evaluations of human specimens. This significant set of in vitro and ex vivo studies which we implement in the laboratory, guide the design of refined in vivo studies. In vivo studies are carefully planned to generate statistically significant data using the minimum numbers of animals. We have also estimated the number of times each protocol will be used based on their usage for the past five years of the present license.

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

The Experimental Design Assistant (EDA) is a free resource from NC3Rs which helps to design robust experiments more likely to yield reliable and reproducible results, thereby reducing animal wastage and making every experiment count. NC3Rs have nationalised the programme allowing access to help by all establishments. Additionally, our researchers under this licence will be kept up to date through the NC3Rs website (https://www.nc3rs.org.uk/the-3rs).

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

Breeding will be carefully planned ahead of each study to allow for the appropriate numbers of animals to be available for experimental procedures and to avoid breeding excess animals. Every effort will be made for any excess animals to be shared with other research groups within our organisation, so that these are not unnecessarily produced. When testing any new hypothesis, we routinely conduct pilot studies, e.g., tumour uptake and growth rate, dose ranging studies, biomarker detection optimisation etc. These studies would be done with small groups of animals prior to a larger, statistically powered experiment. These steps minimise experimental failure and the need for repeat studies. To help generate maximum amount of information from each experiment, tissues will be harvested under terminal anaesthesia or post-mortem at the end of study for further analyses, and these can also further guide future 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 and rats have been chosen as experimental animals as they have the lowest neurophysiological sensitivity while still having an immune system of comparable complexity to the human and are therefore the most frequently used animals in studies of human pathology/immunopathology.

Mouse models involving human tumours have been refined by selection of appropriate cancer models and doses that permit consistent growth of a human tumour xenograft in immunocompromised strains. Mouse models may also be engrafted with human immune cells to make 'humanised' models so that we can understand how our immune system interacts with cancer and we can assess the role of the immune system in the functions of anti-cancer therapies. Other refinements include tumour engraftment into appropriate anatomical locations to generate mouse models of different cancer types such as melanoma, breast and ovarian cancers, allowing the evaluation of cancer growth in an anatomical location where cancer originates or disseminates, and thus these models are more likely to recapitulate the human disease setting

Our rat models have been refined over many years to create highly reproducible models for preclinical efficacy and safety studies of therapeutic interventions. In the context of antibody (IgE/IgG) therapies, rats have immune systems with similar IgE antibody Fc receptor distribution and expression on immune effector cells that closely mirrors that of humans; they are therefore the most clinically relevant animals in studies of antibody function, safety and efficacy. Insights generated from these models have been pivotal in the pre-clinical evaluation of our team’s monoclonal antibody which was tested in patients in a first-in-man clinical trial. Our data generated with these models have shown several parallels with responses to the treatment in patients, supporting the significance of these refined models for the study of a new generation of Fc modified antibodies such as those of the IgE class in future studies.

Potentially therapeutic agents will generally be delivered by different routes such as intravenously. These interventions are needed to allow assessment of therapeutic agents before they can be selected for testing in patients with cancer. A variety of minimally-invasive sampling and measuring methods will be used of which the most severe is blood sampling.

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

Solid tumours such as melanoma, ovarian and breast cancers normally manifest in adults. Therefore, adult rodents have been chosen as experimental animals. Rodents have been selected as they have the lowest neurophysiological sensitivity while still having an immune system of comparable complexity to the human and are therefore the most frequently used animals in studies of human pathology/immunopathology.

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

The protocols used involve least pain, suffering or distress or lasting harm for the animals. None of the intended procedures reach beyond the moderate level of severity. Procedures reaching moderate severity arise from the induction of the disease model (cancer) and are thus inherent to the disease studied. The limits of tumour sizes and endpoints for tumour growth are in adherence with recommendations published by Workman et al., 2010. There are no major invasive procedures except those of skin tumour, normal skin and breast tumour transplantation. These procedures involve well- established surgical techniques.

Anaesthesia and analgesia will be administered to minimise discomfort, and the animals will be monitored closely during all procedures and assessed regularly for any signs of distress. In all the proposed in vivo models, if animals display signs of distress, advice will be sought from the Named Veterinary Surgeon and, if distress cannot be alleviated, the animals will be humanely euthanized.

When it is necessary to conduct in vivo studies, our protocols involve established techniques involving the minimum of suffering. Anaesthesia and analgesia are administered to minimise discomfort, and the animals are monitored closely during all procedures and assessed regularly for any signs of distress. In our in vivo models, if animals display signs of distress, advice is sought from BSF staff, NACWOs or the NVS and, if distress cannot be alleviated, the animals are humanely euthanised. To ensure we use the minimum number of animals in our experiments, we place great care on the experimental design and assessment of the statistical data needed before starting the experiments. We routinely conduct pilot studies in order to determine optimal doses and conditions for our subsequent assessments.

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

The limits of tumour sizes, limits of interventions and endpoints for tumour growth are all in adherence with recommendations published by Workman et al., 2010. The ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines which were developed as part of an NC3Rs (https://www.nc3rs.org.uk/the-3rs) initiative to improve the design, analysis and reporting of research using animals – maximising information published and minimising unnecessary studies.

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

Experimental Design Workshops are run regularly in our institution. The Experimental Design Assistant (EDA) is a free resource from NC3Rs helps to design robust experiments more likely to yield reliable and reproducible results. Additionally, researchers under this licence will be kept up to date through the NC3Rs website (https://www.nc3rs.org.uk/the-3rs).

2. Developing novel therapies and imaging biomarkers for pulmonary hypertension

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

pulmonary hypertension, right heart failure, imaging, therapy, theranostic

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 program of work is to discover new drug targets and develop new therapies for the treatment of pulmonary hypertension, and establish methods to monitor 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?

Pulmonary arterial hypertension (PAH) is a devastating disease that affects the blood vessels in the lungs and leaves sufferers feeling breathless and exhausted. Patients usually die of right heart failure. It is estimated that this disease affects over 60 million people worldwide. Current treatments target only the symptoms and prognosis is very poor. Once diagnosed with PAH, a person has a 50 percent chance of dying within five years. We aim to develop more effective treatments that can tackle the cause of the disease, such as the pathological lung vascular cell proliferation, inflammation and glucose metabolism dysfunction, as well as establish tools such as advanced imaging for assessing the therapy, with the hope to halt or reverse the diseased lung blood vessels and delay the progression of the disease.

PAH arises from the complex and dynamic interaction between multiple cell types in multiple organs that regulate the flow of blood to the lungs, and the subsequent gas exchange processes. It is not currently possible to model all these aspects computer-based or in cell culture settings (including in laboratory cultured mini-organ setting and lung slices) as they lack the ability to fully reproduce the interaction of air and blood within a functioning heart-lung system. We have established rodent pulmonary hypertension (PH) models that recapitulate the genetic mutations and mimic some if not all the distinctive pulmonary vasculature changes and right heart failure process observed in patients. Rodent models allow us to modify single variables (such as a gene), either disease or treatment related, in a controlled environment, which are critical to the understanding of pathophysiology and molecular underpinnings of the human conditions, contributing extensively to therapy and biomarker discovery. We assess pharmacological efficacy of drugs with integral non-invasive multimodality imaging monitoring, alongside measurement of pulmonary artery pressure and right heart pressure; specific tissues can be collected for biological assessment at the defined endpoints. The accessible anatomical, functional, metabolic, and molecular information from rodent models provide invaluable insights into disease mechanisms and mechanisms of drug action, facilitating the later clinical trials of potential therapies in patients.

Our research group is multidisciplinary and includes a clinical team of medical doctors that are able to translate compounds that are successfully developed preclinically into the clinical setting by performing clinical trials in humans. Thus, there is potential for direct impact to PH patients.

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

The ultimate goal of this project is to discover new drug targets and develop new therapies for the treatment of pulmonary hypertension, and establish biomarkers for assessing response to treatment.

In parallel to a clinical program in collecting detailed and comprehensive profiles of PH patients and establishing genetic signatures, we will validate the findings using gene knock out animal models. We have access to pharmacological reagents and experiences with rodent models, as well as a track record in developing new treatments from cell culture through animal models to clinical trials. Our research group is multidisciplinary and includes basic scientists and medical doctors that are able to translate compounds that are successfully developed preclinically into the clinical setting by performing clinical trials in humans. Thus, there is potential for direct impact to PH patients.

The main outputs of this project are:

- Understanding the biology of pulmonary hypertension in living subjects;
- Development of new therapeutic agents, i.e., 1-2 compounds that can be used for treatment of PH - that are successful enough to progress to clinical trials;
- Development of new imaging methods, i.e., 1-2 imaging agents that accumulate specifically in the lung and heart providing better imaging contrast - that are successful enough to progress for use in humans; data modelling to validate the genetic impact observed from patients;
- Publications to increase the existing knowledge about pulmonary hypertension.

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

During the course of this project, we will generate new ideas and advances in understanding pulmonary hypertension that will help drive scientific research in the short term and benefit other research groups in the field. Some of our approaches, have the potential to yield new 'druggable' targets to prevent or resolve the cardiopulmonary pathology, and this may be of benefit to industry (medium-term).The ideas will be tested and the new targets established can be the basis of medical tests and treatments to help patients. In a longer term, we hope the studies on humans and patients with disease will be followed. If these are successful we will be able to turn our basic scientific research into real benefit for patients living with pulmonary hypertension.

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

We collaborate extensively with academic and industry collaborators to ensure we maximise the impact of each study through both publications, and where possible through driving clinical translation. In addition to the publication of full research manuscripts we also actively present our research at the main medical and scientific conferences with our field.

Species and numbers of animals expected to be used

- Mice: 2500
- Rats: 3500

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 aims are to develop new targeted drugs, new blood biomarkers and new imaging agents for pulmonary hypertension patients. A significant part of our work will be done using isolated lung vascular cells grown in the laboratory in cell culture dishes, as well as computer-based approaches. These initial experiments will ascertain relevance of the processes being studied, and permit optimisation. Ultimately, however, animals have to be used to determine whether in the whole organism, the specific biological target is being modulated, and if the sensitivity and specificity or contrast are achieved over healthy organs. Our approach is staged: starting from animal models that are easy to monitor visually to more complex models for which we will add imaging/blood biomarker to clinical

signs for monitoring. We have knowlege on how to establish suitable models of pulmonary hypertension using adult mice (6-12 weeks) and rats (6-14 weeks). The adult mice and rats are the smallest mammals available that possess a cardiovascular system that is sufficiently similar to human, that can be non-invasively and invasively measured to allow the study of the underlying disease processes, and assess the impact of treatments.

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

Typically, we will induce pulmonary hypertension in animals by an injection of a drug, often in the combination with exposure to low oxygen. These models will be used for assessing whether loss/blockade of a gene/proteins protects against/reduces the development of disease over the period of 4 (mice) to 8 (rats) weeks. The choice of compound or exposure to low oxygen to induce disease is guided by the scientific questions being asked and carefully considered to accommodate the gene/protein/drug target being examined. Animals will be monitored carefully throughout the experimental period. Experimental evaluation involves treating the animal with a therapeutic agent for a period of time (by an appropriate route accordingly e.g. oral drinking water or feed with a tude, topical application, injection, apply to nose by liquid drops) and assessing efficacy by imaging animals along the disease process and perform canulation for pressure measurement at the endpoint. On average, the typical animal will undergo the following procedures: 1) non-invasive baseline imaging; 2) disease induction; 3) mid-point pre-treatment imaging; 4) treatment; 5) final end-point imaging,cardiac catheterisation for pressure measurement and tissue harvest. For imaging, the animal will be anaesthetised, have the contrast agent injected in its tail and put inside a temperature-controlled imaging scanner for 1 hour. after the endpoint scan or hemodynamic measurement, the animals will be humanely killed while still under anaethesia so that the heart and lung tissues can be collected to validate the experiment and confirm the accumulation of the compound tested.

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

The most common adverse effect for the animals will be associated with the development of pulmonary hypertension regardless of the model used. Animals present with a general discomfort, loss of appetite, breathlessness and weight loss, similarly to human disease. Models of pulmonary hypertension typically induce disease over a period 3 weeks for mice, and between 3-8 weeks for rats. Rat models usually set 2-6 weeks for disease to develop and further 2 weeks to perform treatment.

Changes in animal welfare as disease progresses are generally subtle in the first instance and slowly progressive. On occasion, a small number of animals can develop a more rapid disease progression and weight loss. Animals are monitored closely throughout (1-2 checks daily) using an animal scoring sheet which assesses body condition, breathing effort, appearance and behaviour (natural and provoked). The scoring sheet enables us to monitor general well being and disease progression closely, and ensure deterioration towards a humane endpoint is identified at the earliest opportunity. Weight loss remains an important indicator of welfare. In addition, pain may be caused during injection of reagents or associated with surgery. These will be minimised with good techniques (e.g. use of smaller needles accurate and slower injection) and application of appropriate analgesia.

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 of these studies is moderate (>95%)for both mice and rats. Rarely, animals undergoing surgery may incur surgical complications in which case they will be humanely killed. All animals will be humanely killed at the end of the study, or if they are deemed to be likely to exceed moderate severity limits.

The expected severities are as follows for both mice and rats

20% - sub threshold

20% - mild

54.5% - moderate

0.5% - severe

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?

No treatment currently in use has been shown to affect structural remodelling of blood vessels in patients.This is challenging as lung biopsies are dangerous in patients with pulmonary hypertension. Tissue samples are extremely limited and only available from patients with end-stage pulmonary disease, either undergoinglung transplantation (rare) or at post-mortem. Animal studies are therefore necessary to study how the disease initiated and developed, and the role that various environmental and genetic factors play in the progression of disease. Understanding the fundamental process involved in pathogenesis through the use of these models is critical for the discovery of new therapeutic targets and the development and testing of new more ‘targeted’ and effective treatments.

In addition animal studies are necessary to evaluate agents that might have therapeutic value in the treatment of pulmonary hypertension. Studies in cell culture alone are not sufficient to provide confidence of a therapeutic effect *in vivo*. This requires an integrated assessment of the pharmacology of the drug and studies in animal models are an important and recognised pre-clinical step. At this time, there is no recognised simulation that will replace in vivo testing in animals.

Prospective pharmacological agents will be assessed first in cell culture experiments, for example using assays of proliferation and cell death to assess efficacy. Only those compounds that are effective in cell culture studies will be considered for further study in animals. Furthermore, non-invasive imaging such as positron emission tomography (PET)

scans is attractive. But the signals detected using PET tracers need to be validated and this can only be done by comparing the information obtained by imaging with the microscopic examination of the tissues obtained from the same animals at autopsy. Once a PET tracer signal is validated, it can then be used to follow drug response in patients with some confidence.

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

All the molecules and reagents that we currently study (as potential treatment) have been initially worked on in vitro using a combination of human (diseased and healthy) and rodent cells to study their role in fundamental processes such as cell proliferation and migration for indicating efficacy. Only those compounds that are effective in cell based studies will be considered for further study in animals. We will also use using blood-derived cells from PAH patients which act as surrogates for human PAH lung cells in our investigation. We have also worked with groups who have currently initiated work on pulmonary artery-on-a-chip (engineered pulmonary artery tissues using pulmonary smooth muscle cells) which will allow testing of drugs on cells from PAH patients.

We have an excellent collaboration with clinical colleagues in the pulmonary hypertension service centres in UK and internationally. We have established biobanks of data (tissue, plasma, imaging) from both human and animal models, these provide us with excellent tools for the discovery of importantnew molecules, cross validation and further study.

Why were they not suitable?

One of the objectives is to identify agents that might have therapeutic value in the treatment of PAH. Studies in cell culture alone are not sufficient to provide confidence of a therapeutic effect in the body, e.g. lacking sufficient complexity to study multiple cell types in a familiar environment, and cells grown on plastic 'behave' very differently than when maintained in the body. Organoids or tissues slices similarly lack the ability to study the complex interaction between blood and air, and again are often performed in a very different environment to that of the whole animal system assessment of the pharmacology of the drug and studies in animal models are an important and recognised pre-clinical step. The usual process is that prospective pharmacological agents will be assessed first in cell culture experiments, for example using assays of proliferation and cell death to assess efficacy. Only those compounds that are effective in cell culture studies will then be progressed for further study in living beings.

In addition, lung biopsies are dangerous in patients with pulmonary hypertension. Histological examination of tissues (lung and heart) from animal models provided confirmation of the impact of the molecules investigated for treating the disease, as well as the validation of the imaging signals.

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 seek advice from statisticians who help 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. Sample sizes for our experiments are estimated from past experiments. Calculations typically show that we need group sizes of 8-10 per group 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. In any given year we typically maintain rat genetically altered lines (1-2 lines) breeding to generate the procedural animals with tissue specific gene (knock- out or insertion of a human gene) accounts for 250 rats per year. These numbers allow us to test the role of 4-6 genes/drugs per year.

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

We have sought and implemented expert statistical advice and support in the experimental design phase to reduce the number of animals being used in this project. Initial experiments will use human and rodent pulmonary artery smooth muscle cells in culture for selection of effective agents and establish dose. Our protocols are structured so that multiple measures can be collected in the same animal (hemodynamic, echocardiography, MRI, PET/CT imaging, tissue collection) along the disease develoment to maximise use and reduce numbers required to answer the scientific questions. At the same time we randomise the animals and run the experiment blindly by separating tasks (dosing, phenotyping and analysis) to particular individuals, hence avoiding bias. We have familiarised ourselves with current guidelines and employed the NC3R's Experimental Design Assistant prior to designing experimental protocols to ensure that rigorous statistical and scientific principles are upheld.

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 seek professional support for ensuring any breeding of genetically altered lines is as efficient as possible. We have optimised the number of breeding animals, and timed matings within each line to generate the required numbers of offspring for experimental procedures. We have also established an AI data analysis program and system for heart imaging which helps to automate the precise and compatible measure for assessing pharmacological reagents. We have also built up the animal tissue bank (from the diseased models) through the years which enabled molecular tests before setting up experiments in animals, and will continue 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.

The rodent models of pulmonary hypertension described in this proposal are recognised internationally as informative for the development of new therapeutic agents for PAH. Typically, pulmonary hypertension in animals will be induced by exposure to low oxygen, or anapplication of a drug (e.g. Sugen to induce endothelia dysfunction, Monocrotaline to induce inflammation, TLR7/8 agonist to induce immune dysfunction, bleomysin to induce inflammation and fibrosis), sometimes in the combination with exposure to low oxygen. Genetically altered animals will also be used to characterise the phenotype of a gene or physiological and pathological importance of a particular genetic elements, e.g. models for recaptulating the genetic mutations (e.g. BMPR2, SOX17) discovered in patients. The PH animal models are the best characterized models currently available for mimic some if not all the distinctive pulmonary vasculature changes and right heart failure process observed in patients. These models have their limitations and results have to be interpreted accordingly. All current therapies for PAH have been examined in these models and so there is a benchmark against which to judge the potential value of new agents. According to our previous experience (of more than 30 years) as well as that of our collaborators and with improved the detailed protocols and techniques, these animals models of pulmonary hypertension usually tolerate all the insults with little evidence of severer illness within the proposed study period, mortality is <1% within our experimental setting.

The efficacy of treatment will be assessed by measuring the change in pulmonary artery pressure, the change in right ventricular mass, the degree of vascular remodelling and, in the all the animals disease development process, time to ill health – defined by agreed criteria (by implementing a species specific animal welfare scoring system, assessment for signs of ill health and killed humanely to avoid unnecessary suffering). By developing and promoting the PET and MRI imaging we will be able to assess the early stage of cell proliferation and vascular remodelling in all the models.We work closely with the named Veterinary Surgeon and animal care staff to ensure that the procedures and animal husbandry conditions are as refined as possible. Animals will be housed in groups where possible, with appropriate environmental enrichment and fed according to current institutional 'best practice'.

To induce gene expression in animals or to deplete specific cells, some animals will be given substances by mouth, injection, or through food. Oral gavage or injection are necessary to assess the pharmacological reagents. Animals will be treated using methods similar to human, e.g., injection of substances under the skin, into the abdomen cavity or by intravenous injection.

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

To fully study the disease processes in humans we require adult (already developed) mammalian cardiopulmonary system that can be 'easily' manipulated to study the functional role of molecules in disease development. The nature of the disease requires multiple techniques to diagnose humans with the disease, and we employ similar methods in rodents. We use the smallest available pressure catheters to measure heart pressures in mice so are limited by size in smaller, or younger animals.

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

We will ensure that team members receive comprehensive training on animal welfare principles, behaviour, and proper handling techniques, as well as attending regular workshops on the animal research and best practices. A routine monitoring score sheet

will be used to assess animal welfare indicators such as behavior, health, and physiological parameters accordingly (see the following score form). This allows for early detection of any issues or signs of distress, enabling prompt intervention. Weight loss remains an important indicator of welfare. Where any mouse or rat records a loss of >10% either from their starting weight, or from their previously recorded weight, twice daily checks using the scoring sheet and daily weights are recorded until the rodent gains weight on 2 consecutive days.

We will review and refine experimental procedures continuously to minimize any potential distress or discomfort experienced by animals. For example, when giving treatment, we will try and use diet over oral gavage wherever possible. We will ensure appropriate measures are in place to alleviate pain and discomfort associated with experimental procedures or any health issues. This may involve the use of analgesics, anaesthetics, as well as techniques such as local anaesthesia or numbing agents. All the experiments will be planned and recorded carefully, also ensuring the procedures are in compliance with relevant regulations and guidelines. We encourage team members to work closely with veterinarians, animal care staff, and regulatory staffs to exchange knowledge and experiences regarding best practices in animal welfare. By implementing these strategies and fostering a culture of care and responsibility towards animals used in research, it is possible to minimize welfare costs and uphold ethical standards while advancing scientific knowledge.

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

We will follow published guidelines to assist with planning animal research and testing, such as the PREPARE guidelines:http://journals.sagepub.com/doi/full/10.1177/0023677217724823 and https://norecopa.no/prepare.

We will also make use other resources that are available including guidance and publications from 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 check information on NC3Rs website, we've signed up to the NC3Rs newsletter and we will attend regional 3Rs symposia.We also share best practice with both industry and academic collaborators though conferences and research meetings, actively looking for ways to implement any improvements for in vivo experiments.

3. Understanding the neural regulation of central and peripheral circadian rhythms

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

Circadian rhythms, Sleep, Ageing, Mental Health

Animal types	Life stages
Mice	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 investigate the neural mechanisms, from behavioural input to cellular physiology, that drive the mammalian circadian clock.

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?

Investigating the neural mechanisms of the mammalian circadian clock is important as circadian rhythms are involved in multiple aspects of our lives, contributing to health and disease. For example, disruptions to the circadian rhythm are linked to sleep disorders like insomnia and shift work sleep disorder. By understanding the neural mechanisms, we can develop better treatments to regulate sleep patterns. Circadian rhythm disturbances are also implicated in mood disorders like depression. Our research can aid in developing therapies targeting the clock for improved mental health.

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

We will publish our findings in the open access scientific journals, we will work closely with healthcare providers to implement our findings in practice (such as in care homes).

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

Our findings will benefit the wider scientific community, it will improve the understanding about circadian rhythms and how they can affect our wellbeing and what changes happen as we age. In the longer term, our work will have a direct input on patients, such as those with mental health disorders or shift workers.

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

We will disseminate our findings with the wider community through public lecture series that we have at the University, will present findings at specialist scientific conferences across the world (either successful, or unsuccessful work), will share our findings on social media, such as LinkedIn, to increase awareness of the work we do.

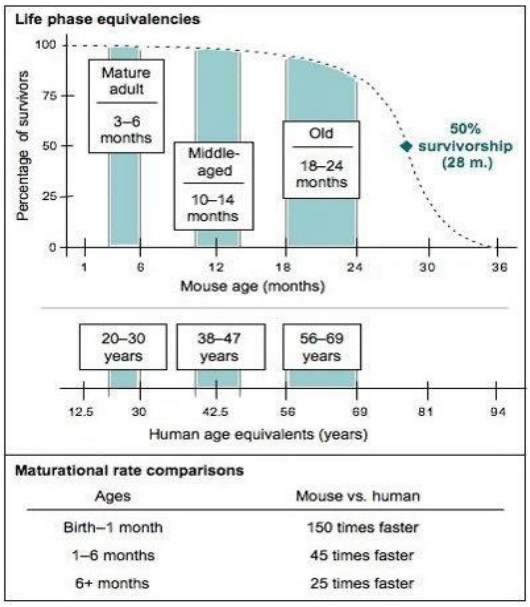
Species and numbers of animals expected to be used

- 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.



Maturational rate comparisons

Ages	Mouse vs. human
Birth–1 month	150 times faster
1–6 months	45 times faster
6+ months	25 times faster

The use of mice in our circadian research is driven by several compelling reasons related to both the practicalities of scientific investigation and the biological relevance of these animals. Mice are a well- established model organism for studying circadian rhythms, allowing researchers to conduct detailed and longitudinal studies that would be highly

challenging to perform in humans. These animals offer a significant advantage due to their relatively short lifespan and well-characterized biology, making them ideal for examining changes across the entire life span within a manageable timeframe.

For most of our experiments, we use mice that are 3-6 months old. This age range corresponds to young adult mice, analogous to humans aged 20-30 years. Using mice at this life stage enables us to investigate circadian rhythms and behaviours in mature but not yet aged organisms, providing insights into adult physiology and behavioural patterns that can be extrapolated to similar stages in humans.

In our research focused on ageing, we extended the age of the mice to 18-24 months as well as 24-36 months. Mice in this age range are comparable to humans aged 56-69 years and 69-94 years. Studying mice at this older age allows us to explore the effects of ageing on circadian rhythms, offering valuable data that can help us understand how ageing impacts biological and behavioural processes.

This approach is crucial for identifying potential interventions or treatments that may mitigate age- related disruptions in circadian rhythms in humans.

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

In this project, the procedures involving animals primarily focus on behavioural and physiological studies that will include activity monitoring using passive infrared sensors, housing under variable light- dark cycles, behavioural testing, such as maze tests, light/dark box tests, responses to light pulses or pharmacological compounds mimicking the effects of light. For studies requiring drug or viral vector application directly into the brain, animals will have a cannula implanted into the brain or receive a single injection during the stereotaxic surgery. The animals will be subject to carefully controlled environments to facilitate the recording of their behaviour and physiological responses. One aspect of the study involves housing the animals individually. This is necessary to ensure accurate behavioural recordings using passive infrared sensors. These sensors are designed to detect and record the movements and activities of the animals without any direct interference or disturbance.

A significant component of the project includes manipulating the light-dark cycles under which the animals are maintained. Unlike the standard 12-hour light and 12-hour dark cycle, the animals may experience altered cycles, including extended periods of light, extended periods of darkness, or constant light or dark conditions for no more than 6 weeks at a time. These adjustments will be implemented either in the general animal housing rooms or within specialized light-tight chambers. These chambers are ventilated and sound-attenuated to provide a controlled and undisturbed environment for the animals.

In addition to behavioural monitoring, a substantial portion of the project will involve recording electrical activity from the animals' brains. This procedure is critical for understanding the neural underpinnings of their behaviour and cognitive processes. Alongside neural recordings, the project will also measure gene expression from tissue samples. These measurements will help in identifying the molecular changes associated with the different experimental conditions and behaviours observed.

The behavioural experiments will generally span a period of 3 to 6 months. Throughout this time, the animals will be monitored and maintained under the specified conditions to

ensure their well-being and the integrity of the data collected. All procedures are designed with consideration for the animals' health and comfort, adhering to ethical standards and regulations governing the use of animals in research.

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

In the course of this project, the majority of procedures will have minimal to no adverse effects on the animals involved. The primary impact anticipated is comparable to the symptoms humans might experience during jet lag, such as temporary disruptions in sleep patterns and minor changes in activity levels. These effects are typically transient and are expected to resolve as the animals adjust to the altered light-dark cycles.

For procedures involving injections, the animals might experience initial discomfort at the injection site. This discomfort is usually brief. A subset of the animals may undergo surgical procedures as part of the study. The potential adverse effects associated with these surgeries include a temporary decrease in food and water intake, poor wound healing, and in some cases, detached sutures. These effects are monitored closely, and comprehensive postoperative care (such as daily body weight measurements) is provided to alleviate any issues. This care includes the administration of sterile solutions to prevent dehydration, gel supplements, and moistened diets to encourage appetite and hydration.

In studies involving aged animals, the observed effects will largely stem from natural ageing processes. Common age-related symptoms in these animals may include hair loss or overgrooming, increased body weight, and deterioration of organ function, such as impaired vision. These conditions are managed as part of routine care, with specific interventions to ensure the well-being of the aged 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)?

In this project, the expected severities of the procedures and the proportion of animals falling into each category have been carefully considered to ensure the welfare of the animals while achieving the research objectives. The majority of the animals used in our studies are expected to experience only mild severity. This is primarily due to the nature of the procedures they will undergo, which mainly involve changes in their light-dark cycles. These alterations are akin to the effects of jet lag in humans and do not involve significant pain, distress, or lasting adverse impacts. Approximately 70% of the animals in our project will fall into this mild severity category.

A smaller proportion of the animals, specifically those that are aged or those undergoing surgical procedures, will be categorized under moderate severity. This group is expected to comprise about 30% of the animals used in the project. The aged animals are housed for extended periods to observe the natural ageing process and its effects on circadian rhythms. While these animals are not subjected to any invasive procedures solely due to their age, the prolonged housing and natural ageing process itself can lead to conditions such as hair loss, overgrooming, increased body weight, and deterioration of organ function, including vision.

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 in this project is essential to achieve our research objectives, which focus on understanding the underlying mechanisms by which the circadian system is controlled and how it is affected by ageing. This research requires an intact physiological system, something that cannot be replicated by less complex models or in vitro studies.

Circadian rhythms are inherently linked to the functioning of complex biological systems, including the brain, endocrine system, and various peripheral organs. These systems interact in intricate ways to maintain the body's internal clock. To study these interactions comprehensively, it is necessary to work with whole organisms where these systems are fully integrated and operational. For instance, the role of the eyes in decoding light information and transmitting it to the circadian clock is a crucial aspect of our research. The eyes contain specialized photoreceptor cells that detect light and convey this information to the brain, which in turn regulates circadian rhythms. Such a process involves multiple layers of complexity that cannot be adequately studied in isolated tissues or less complex organisms.

Moreover, our work involves the use of genetically modified animals to dissect the roles of specific genes and molecular pathways in the regulation of circadian rhythms. These genetic modifications allow us to observe the effects of altering or deleting particular genes within the context of a whole, living organism. This approach provides insights into the genetic components of the circadian system that would be impossible to obtain using simpler models.

Using animal models, particularly mice, also enables us to conduct longitudinal studies and observe the effects of ageing on the circadian system. The ageing process involves a myriad of physiological changes that impact multiple systems simultaneously. Studying these changes requires an organism with a complex physiology akin to that of humans. Mice are particularly suitable for this purpose due to their well-characterized genetic makeup and the availability of age-equivalent stages that correlate with human ageing.

To minimise the use of mice and optimise our research methods, we will also be working with Drosophila (fruit flies) as an initial model. Drosophila offers several advantages – simpler genetic structure allowing easy manipulation and testing of hypotheses to better understand mechanisms underlying circadian rhythms. By using fruit flies to identify key genetic and molecular pathways, we can design more targeted and efficient studies in mice, reducing the number of mice used.

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

We considered using cell cultures.

Why were they not suitable?

While cell cultures can offer valuable insights into specific cellular processes and molecular mechanisms, they fall short in providing the comprehensive understanding required for our study of the circadian system and its regulation.

Cell cultures allow for the manipulation and observation of isolated cells in a controlled environment. They are particularly useful for studying cellular responses to various stimuli, gene expression, and protein interactions at a detailed level. However, circadian rhythms are governed by complex interactions between multiple organs and systems within a whole organism.

Furthermore, our project involves studying the effects of ageing on the circadian system. Ageing impacts multiple interconnected physiological systems, leading to changes in circadian rhythms and behaviours. To understand these age-related changes, it is necessary to observe the interactions between the central and peripheral components of the circadian system in an intact organism.

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 of the number of animals required for this project has been carefully determined based on extensive experience and historical data from our research over the past decade. We have drawn on data from our previous studies to estimate the number of animals needed for breeding transgenic lines. Breeding transgenic animals is essential for our work, particularly given our focus on genetically modified models to understand the circadian system. Our past projects have provided a robust framework for predicting the breeding success rates and the proportion of animals that will express the desired genetic traits. This historical data helps us to estimate the number of breeding pairs required and the expected yield of transgenic offspring.

Furthermore, the number of animals needed for the experimental procedures has been estimated based on the requirements of our research protocols and the specific scientific questions being addressed. Our previous work has given us a clear understanding of the number of animals necessary to observe meaningful differences in circadian rhythms and behavioural responses under various experimental conditions. We have also considered the duration and complexity of our studies, ensuring that each experimental group is appropriately sized to yield statistically significant and reproducible findings.

Additionally, we have reviewed the practices of other research groups conducting similar studies. This benchmarking provides valuable insights into standard animal usage for comparable experiments. By aligning our estimations with those of established practices in the field, we ensure that our approach is both scientifically rigorous and consistent with current research standards.

Finally, we have incorporated consideration for contingencies, such as potential breeding inefficiencies, unexpected experimental variability, and the need for control groups. This conservative approach ensures that we have a sufficient number of animals to achieve reliable results while avoiding unnecessary surplus.

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

When designing experiments, we followed the principles of NC3Rs and used Experimental Design Assistant (EDA). This resource provided valuable guidance on designing experiments in a manner that maximizes the information gained from each animal while minimizing the overall number needed. By leveraging the EDA, we were able to optimize our study designs, identify potential sources of variability, and calculate appropriate sample sizes to achieve statistically robust results.

Furthermore, we have a number of years of experience undertaking behavioural research and we have refined our experimental protocols and procedures. These investigations allowed us to identify any potential issues or inefficiencies early on and make necessary adjustments.

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 practices play a crucial role in minimising the number of animals used in our research. We have established breeding strategies that are carefully managed to ensure optimal reproductive success and the generation of the desired experimental cohorts. We also use males as well as females in all of our experiments, minimising the waste of animals.
To increase the benefits of the animals we use, we actively pursue opportunities for the sharing and reuse of tissue samples wherever possible. By collaborating with other research groups within our department and external colleagues, we contribute to the generation of new insights without the need for additional animal 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.

In our project, we will use specific animal models and methods tailored to investigate the intricate mechanisms underlying circadian rhythms and their interaction with ageing. In addition to standard wild-type mice used in circadian research, we will use animal models with selectively deleted proteins involved in circadian control, such as NR2B. These genetically modified mice allow us to elucidate the roles of specific proteins in regulating circadian rhythms and understanding their impact on behaviour and physiology.
Importantly, the circadian disruption animals we use do not exhibit harmful traits that might

cause pain or suffering, ensuring ethical treatment throughout the duration of the study.

One innovative transgenic animal model we employ is designed to mimic age-related changes in circadian rhythms without the need for prolonged housing. Using the Cre-Lox system, we created conditional NR2B knockout animals, where the NR2B gene is specifically deleted in circadian clock neurons. This method allows us to induce an aged genotype in these neurons at a younger age, minimising the need to house animals for extended periods until they naturally age. Additionally, the conditional knockout approach protects the animals from the harmful phenotypes that would occur if they were bred as full knockouts. This refined method not only accelerates our investigations into the effects of ageing on the circadian system but also adheres to high ethical standards and welfare considerations.

Furthermore, we ensure the validity and reliability of our experiments by using appropriate controls. These controls have the same genetic modifications as the NR2B knockout animals but retain the NR2B protein, allowing us to distinguish the specific effects of NR2B deletion.

Most of our work is conducted using non-invasive interventions to further prioritise animal welfare. These methods include altering the light-dark cycle and using behavioural recordings with infrared sensors. Such approaches enable us to study the natural behaviour and physiological responses of the animals without causing distress or discomfort.

By integrating these refined techniques and ethical considerations, our research aims to provide valuable insights into the regulation of circadian rhythms and their alterations with ageing, while minimising the use of animals and ensuring their humane treatment throughout the study.

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

In our research project, the use of animals with a fully developed circadian system is indispensable. While it may be tempting to consider using animals that are less sentient for experimental purposes, doing so would severely compromise the validity and reliability of our findings. The circadian system's complexity involves the interaction of multiple systems, including the brain, endocrine system, and peripheral organs, all of which are intricately linked to maintain the body's internal clock. Studying these interactions requires an intact, fully developed physiological system that simpler organisms cannot provide.

We will be using Drosophila (fruit flies) to understand basic mechanisms of circadian rhythms. Drosophila serves as an excellent initial model due to its genetic simplicity and the ease with which it can be manipulated. Insights gained from studies with Drosophila will guide and refine our experiments with more complex animal models. However, to gain comprehensive insights into how ageing affects circadian rhythms and vice versa, it is crucial to study these processes in mammals. Mammals have circadian systems that closely mimic human physiology and ageing trajectories, providing the necessary complexity to understand the underlying mechanisms and their implications for health and disease.

Moreover, the ageing process itself involves intricate changes in circadian rhythms and physiological functions. To gain accurate insights into how ageing impacts circadian rhythms and vice versa, it is essential to study these processes in animals that can

replicate the physiological changes occurring in ageing humans. Mice, for instance, are particularly valuable due to their well-characterised genetic makeup and similarities to human ageing stages.

Additionally, we prioritise the welfare and well-being of the animals involved in our research. Whenever possible, we perform studies ex vivo using tissue samples only, thereby minimizing distress and reducing the need for live animal experimentation. This approach helps ensure that our research is conducted ethically and responsibly, balancing scientific objectives with the highest standards of animal welfare.

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

One key aspect of our refinement strategy is the use of non-invasive techniques whenever possible. We prioritize methods that do not require invasive procedures or cause undue stress to the animals. For example, behavioural recordings using passive infrared sensors allow us to monitor the animals' activity patterns without direct intervention or disturbance, minimizing any potential welfare costs associated with handling or restraint.

Furthermore, we have optimised our housing and husbandry practices to promote the animals' physical and psychological well-being. Our animal facilities adhere to stringent standards of care, providing standard housing conditions with temperature controlled rooms and daily monitoring. We ensure that the animals have access to appropriate food, water, bedding, and environmental enrichment to support their behavioural and physiological needs.

One significant refinement in our procedures is the use of light-tight boxes with individually controlled light-dark cycles. This setup allows us to precisely control the environmental lighting conditions for each animal, minimizing stress and ensuring that the circadian rhythms are accurately maintained and studied.

Our ageing animals are carefully and regularly monitored for adverse effects such as changes in weight, dermatitis, piloerection, paleness, changes in mobility, lumps, eye defects, abnormal respiration, or stools by staff trained to work with ageing animals. Group sizes in ageing experiments are increased to accommodate for the loss of animals and to avoid single housing due to animal losses due to old age.

In cases where invasive procedures are necessary, such as surgeries or injections, we have implemented refined protocols to minimize pain and distress for the animals. This includes the use of appropriate anaesthesia, analgesia, and postoperative care to alleviate discomfort and promote recovery. We closely monitor the animals' health and behaviour throughout the experimental procedures, adjusting our protocols as needed to ensure their welfare is prioritized at all times.

Before conducting any Animal Behavioural Testing (ABT), we ensure that the animals (and staff) are adequately trained and acclimatised to the testing procedures. This approach minimises stress and allows the animals to become familiar with the testing environment and equipment.

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

We are committed to conducting our experiments in the most refined manner possible, minimising any potential distress experienced by the animals. Therefore, we will adhere to the following published best practice guidance:

- National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs)
- ARRIVE Guidelines (Animal Research: Reporting of In Vivo Experiments)
- Scientific journal publications

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

We are committed to implementing the principles of Replacement, Reduction, and Refinement (3Rs) throughout this project. We will ensure to continuously learn by monitoring relevant scientific literature, will subscribe to newsletters and resources from organizations dedicated to promoting the 3Rs, such as the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs), we will dedicate a portion of our regular team meetings to discuss the 3Rs and their potential application within the project and maintain open communication with the NACWO throughout the project. Furthermore, when new 3Rs methods relevant to our research become available, we will assess their feasibility within our project framework and we will continuously evaluate our existing protocols and identify opportunities for refinement.

4. Vaccines to protect against Nipah virus

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

vaccine, pig, Nipah virus, Pseudorabies, Immunogenicity

Animal types	Life stages
Pigs	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?

To develop a safe and effective vaccine to prevent and aid control of Nipah virus outbreaks in pigs.

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 Nipah virus (NiV) poses a significant epidemic threat to South and Southeast Asia because of its broad host range and widespread distribution of fruit bats which act as a natural reservoir. Humans may become infected indirectly from bats or through exposure to infected pigs or other livestock species. Pig-to-human transmission was responsible for the first and still most severe NiV outbreak in Malaysia and Singapore in 1998-99. Despite the risk NiV poses, no vaccines are currently licensed for humans or pigs. NiV is listed as a priority pathogen by the World Health Organisation for research and development in the context of public health emergencies. NiV infection of pigs is also a “listed disease” notifiable to the World Organisation for Animal Health. The rare and sporadic nature of NiV outbreaks means that a vaccine for pigs has limited marketability. To overcome this

challenge, we aim to develop a bivalent vaccine, which would induce immunity to both NiV and the pseudorabies virus (PrV), which is widely vaccinated against in Asia. If successful, such a vaccine would reduce the risk of NiV outbreaks in pigs and the concomitant severe socioeconomic consequences and threat to public health.

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

Proof-of-concept that a PrV vector expressing NiV glycoproteins induces immune responses to both viruses, including responses that protect pigs against PrV.

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

The scientific community will benefit from the improved knowledge of the performance of a new vaccine approach. This could lead to the development of a safe and efficacious bivalent vaccine that results in prevention and control of NiV and PrV outbreaks, and consequently improved animal welfare, improved productivity in the pig industry, and reduced risk to public health. 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 and reduced incidence of zoonotic disease.

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

All outputs from this project will be published in Open Access scientific journals; this will include unsuccessful vaccine approaches. Outputs of this work will also be disseminated to other stakeholders and the general public through press releases, presentations at meetings/congresses and social media channels.

Species and numbers of animals expected to be used

- Pigs: 39

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.

Pigs are the only natural host for PrV and can act as an amplifying host for NiV. Since we aim to develop a bivalent vaccine to protect pigs against both viruses, they are the most suitable animal to evaluate vaccine immunogenicity and efficacy.

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

Typically, pigs used in this project will be immunised by injection of a live attenuated PrV vaccine candidate into the muscle. This will typically 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. Vaccinated and unvaccinated animals may be challenged once by administration of virulent PrV into the nose. Blood samples and nasal swabs will again be taken at intervals to quantify levels of challenge virus and immune responses. This will typically be done twice weekly. Animals will then be culled humanely to assess tissue pathology and tissues will be collected to assess PrV loads and for further

analysis of immune responses. The typical duration of an experiment is 42 days. Pigs will not be challenged with NiV within this project and instead blood samples will be collected from vaccinated pigs to determine levels of NiV-specific neutralising antibodies. The ability of vaccine candidates to protect pigs against NiV will be assessed by a European partner due to the requirement for a Biosafety Level 4 Biocontainment facility suitable for housing pigs, which the UK does not possess.

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

No clinical signs are expected in vaccinated pigs following vaccination or PrV challenge. Unvaccinated pigs are expected to develop clinical signs following PrV challenge. This is most likely presented as a rise in body temperature from 2 days post-infection. Pigs may stop eating and become reluctant to get up unless touched, or start to develop clinical signs of respiratory and neurological disease. 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 to prevent further suffering if humane endpoints are met. The impact of blood sampling, swabbing and inoculation of vaccine or challenge 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 expected severity for pigs that are vaccinated and pigs that are vaccinated and challenged is mild. The expected severity for control pigs that are unvaccinated and challenged is moderate.

It is estimated that 85% of pigs will be in the mild severity category and 15% of pigs 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 vaccines are protective 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 and characterise vaccine candidates, cultivate vaccine and challenge virus strains and to evaluate virus-neutralising antibody

characteristics. NiV glycoprotein expression by recombinant PrV vectors will be confirmed in cell culture prior to their evaluation 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, need 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 or from relevant published literature 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?

Statistical analysis of data collected from previous related studies. 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?

Use of in vitro models to characterise novel PrV vaccine constructs. Basing study design on recently conducted relevant 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.

Pigs are the natural hosts for PrV and are the target species for a bivalent PrV-NiV vaccine. Whilst PrV has a broad host range, PrV strains that are attenuated in pigs may still cause a fatal infection in other species e.g., mice. The pig is therefore the most suitable animal to study the effectiveness of live attenuated PrV-based vaccine candidates.

A well characterised live attenuated PrV strain will be selected to be engineered to express membrane anchored NiV glycoproteins. We have previously evaluated live attenuated PrV strain expressing soluble forms of NiV glycoproteins - this vaccine candidate was safe, induced PrV-specific immune responses comparable to the parental PrV vaccine, but the immune responses to the NiV glycoproteins could be improved.

Vaccinated and unvaccinated control pigs will be challenged with a well characterised virulent PrV strain. This enables us to assess protection against clinical disease as well as by reduction in virus loads.

Animals will be inoculated with vaccine or challenge virus in the smallest volume commensurate with the aims of the procedure.

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

Pigs are the natural hosts for PrV and are the target species for a bivalent PrV-NiV vaccine. Whilst PrV has a broad host range, PrV strains that are attenuated in pigs may still cause a fatal infection in other species e.g., mice. The vaccine approach has already been evaluated in pigs and was shown to be immunogenic so there is no value in using other less sentient animal models.

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

Animals will be housed together with bedding and other items of enrichment. Highly trained animal technicians will monitor these animals throughout the day, ensuring they are comfortable and to maximise their welfare status. We have 24/7 CCTV surveillance which can be used to monitor the animals’ behaviour over time.

Pre-study meetings involving the Named Veterinary Surgeon (NVS), Named Animal Care and Welfare Officer (NACWO), and animal services staff will be held to discuss any advances in animal care. Meticulous records will be kept of behavioural, physiological, immunological, and virological measures in order to identify predictive markers and refine humane endpoints. 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?

Adherence to the ARRIVE guidelines for reporting these studies, as well as reference to the Federation of European Laboratory Animal Science Associations' (FELASA) guidelines for pig health monitoring to help ensure the most robust health assurance for animals used in this study. FELASA guidelines for administration of substances has been used to limit the maximum volumes for each of the routes.

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

Through continued continued professional development (CPD) and frequent review of the Center for Alternatives to Animal Testing (CAAT) and the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) websites, I will keep informed about advances in the 3Rs. Included in CPD will be annual attendance at

relevant science conferences.

5. Characterising the behavioural phenotypes and neural circuits supporting sense of agency in mouse models

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Sense of agency, Sense of control, Neuroethology, Schizophrenia, Avolition

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 the sense of control is computed through interactions between brain areas during natural behaviours in normal health and psychiatric 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?

The sense of agency (SoA), or the feeling of control over our actions and their outcomes, is a central component of healthy human experience. Although some human studies have made some progress, SoA neuroscience is still in its infancy with limited non-human animal models available to study the neural circuits during natural behaviours. SoA is significantly perturbed in diseases such as schizophrenia disorder (SCZ) and depression where disruptions in cognition, emotion, and behaviour which lead to impairments in personal and social function [1][2].

Advancing our understanding of SoA requires a better understanding how multiple neural

areas in mammals are engaged during natural decision making, motivated behaviours and self-initiated tasks - and how this coordination supports SoA. Mouse models provide the ability to record multiple brain areas during naturalistic behaviours and facilitate SoA research in ways that are not yet possible in humans.

[1] Garbarini F, Mastropasqua A, Sigaudo M, Rabuffetti M, Piedimonte A, Pia L, Rocca P. Abnormal Sense of Agency in Patients with Schizophrenia: Evidence from Bimanual Coupling Paradigm. Front Behav Neurosci. 2016 Mar 9;10:43. doi: 10.3389/fnbeh.2016.00043. PMID: 27014005; PMCID: PMC4783405.

[2] Vogel DHV, Jording M, Weiss PH, Vogeley K. Temporal binding and sense of agency in major depression. Front Psychiatry. 2024 Apr 5;15:1288674. doi: 10.3389/fpsyt.2024.1288674. PMID: 38645414; PMCID: PMC11027068.

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

We will gain new knowledge towards a better understanding of how sense-of-agency is represented by the neural circuits in the mammalian brain in both normal health and mouse models of psychiatric diseases. We will seek to explain components of functional connectivity, i.e. how brain areas interact in vivo, in critical areas such as:

- frontal cortex - responsible for executive function and decision making and implicated in sense of agency.
- motor cortex - responsible for preparing and generating movement and involved in preparation of voluntary action.
- sensory cortex - receiving top-down sense-of-agency attenuation signals.
- insular cortex - responsible for integrating self-representation signals.

Our findings may help identify candidate brain areas for targeting of pharmacological or direct interventions for alleviation of symptoms of psychiatric disorders.

Our work will be released in publications and presentations to the scientific community and public.

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

Our work will benefit our research group and the wider neuroscience community who are seeking to develop models of sense of agency.

Identifying neural mechanisms and behavioural phenotypes of mouse models SoA will help advance our understanding of the SoA in health and consider future applications to schizophrenia.

Our neuroethological approach focuses on collecting data from the fewest number of animals for extended periods of time. This reduces the overall number of animals we use and may also pave the way for other researchers to reduce their animal use numbers.

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

We will seek to collaborate with researchers and other basic neuroscience groups to integrate frontier knowledge of the field into our work. We will also seek to publish both positive and negative results of our work to the broader community.

We will seek to publish some of our work in open-access journals and make some of our datasets publicly available including behaviour videos and neural recording datasets.

Species and numbers of animals expected to be used

- Mice: 280

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 seeking to understand the neural circuits of sense of agency in the mammalian brain. Mice have been validated for use in self-causality tasks by our own research group and others, and there are well established genetic-knockout models of schizophrenia that are readily accessible and could be implemented in the future. Mice are the most appropriate and least sentient non-human animal model that we could use to elucidate the neural circuits of sense of agency in the mammalian brain.

We propose to use wild type mice (C57BL/6J) of both sexes and our criteria for inclusion into the study will be a minimum age of 8 weeks and a body weight of minimum 20-25 grams prior to surgery.

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

Some of the mice will undergo surgery to implant extracellular electrodes for the recording of single neuron and population activity during spontaneous and natural behaviours.

The surgical implants will be performed when the mice are a minimum of 8 weeks old using aseptic surgical techniques. Mice will be given continuous gaseous anaesthetic and fixed onto a stereotaxis platform to hold their head in place. During the surgical procedure, small burrow holes will be made in the skull, probes will be implanted and the wound will be closed with dental cement and appropriate tissue glue. These surgeries will typically last up to 3 hours each.

Mice will undergo post operative care including the administration of analgesics for 3 days after surgery. They will be monitored and weighed daily post-surgery to ensure they are recovering well, eating and drinking normally and score sheets with body weights and behavioural markers will be completed during this period.

Mice will have optional access to tasks such as nose-poking which will provide treats such as sugar pellets. Periodically during each day mice may be restrained for between 30 to 60 seconds at a time, to replace the neural logger batteries which are attached via magnets or connectors to the neural logger. To facilitate interactions, mice will be habituated to handling before and after the surgery using positive reinforcement training protocols emphasising gradual, positive interactions, such as using sugar treats to foster acceptance of handling. For example, mice will be introduced to handling through familiar objects like tubes, followed by cupping, with each stage gradually extended while being provided with food rewards.

Neural activity will be recorded for as long as the implants are viable, and the mice are healthy up to the age of 18 months. At the end of the recording period mice will be perfused under terminal anaesthesia.

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

Animals will undergo implant surgery under general anaesthesia but are expected to make a rapid and unremarkable recovery from the anaesthetic within two hours. None of the surgical intervention or implants is expected to lead to long-term harm.

Mice will be restrained for the changing of neuro logger batteries which will cause stress to the animal but usually only during the first few sessions while the animal is acclimated. Once the animals are used to the restraint, most of the time animals do not show signs of stress and behave normally, e.g. they will groom themselves and nest build.

Animals that develop infections around the implant site will be assessed by the NVS/NACWO and either removed temporarily from the arenas for recovery in an IVC or euthanised by Schedule 1 method. Mice with implants that degrade or become detached and cannot be repaired or replaced will be sacrificed using a Schedule 1 method or perfused 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)?

Approximately 90% of animals will experience moderate severity. These mice will undergo recovery surgical procedures (AB).

Approximately 10% of the mice will experience mild severity harm. These mice will undergo surgical procedure as non-recovery (AC).

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 require the use of mammals to understand how functional interactions between areas of the mammalian brain give rise to the sense of agency and how it is perturbed in diseases such as schizophrenia. The neural computation of sense of agency is complex and in vitro or cell cultures are inadequate models for this complex executive capacity. In vivo multi-area neural recordings in freely behaving animals will allow us to test theories of agency which propose specific neural areas are involved, the direction of their causal interaction and how these relationships degrade in disorders.

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

We have considered alternatives, but these are not feasible to generate the inter-area mammalian brain neural dynamics we require. In particular computational models of behaviour such as reinforcement learning agents - do not have mammalian neuro-anatomical homology nor develop schizophrenia or depression-like behavioural symptoms. Existing databases do not contain self- causality tasks in open arenas and free behaviour in mice.

Why were they not suitable?

Using in-vitro methods, computer modelling, using non protected species or humans or epidemiological data are not suitable alternative methods for our study. Animals must be used for this project as we are interested in understanding the neural dynamics supporting a sense of agency during naturally occurring and innate driven behaviours. The field lacks an understanding of the neural dynamics and causal pathways during such actions and we can only gain this knowledge by recording the neural activity from freely behaving 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?

Our study employs enriched home arenas to capture longitudinal data from naturally behaving mice. Due to the variability in natural behaviours and the complexity of simultaneous recordings from multiple brain areas, this increases the number of mice required. To minimise variance and reduce the number of mice, we aim to gather more data from each individual mouse, where variability is relatively consistent. Based on previous research, only 1% of self-initiated natural actions can be isolated from motor confounds. Rodents exhibit 10 to 100 behavioural events daily, and we estimate requiring 500 to 1,000 trials per behaviour per mouse. With 10 to 100 trials per day, we expect to collect 6 to 12 hours of neural data daily, over 10 to 50 days per mouse. To maximise data collection and reduce animal numbers, we will continue recording from healthy mice up to 18 months old.

We plan to use 8 groups of 30 wild type mice (C57BL/6J), each implanted with neural devices targeting different brain regions central to our study (e.g., frontal, motor, sensory areas, hippocampus, and amygdala). Wireless neural loggers will record from multiple areas in 8 configurations to capture neural activity during behaviours triggered by external stimuli or self-initiated. We estimate requiring 10 implanted animals per configuration to support our analysis. Based on previous experience with the variance in targeting multi-area neural implants we anticipate requiring three times as many mice, i.e. a total of 30 mice per configuration. For the eight recording configurations we will thus require a total of 30 x 8 = 240 wildtype mice.

We will additionally use dye injection studies to test the variance in our anatomical targeting. For this we will use at most 5 mice for each of 8 anatomical areas as a total of 40 mice.

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

We designed our experiments and research paradigm to leverage recording of experimental data longitudinally thus reducing the number of mice required compared to standard approaches that record for several hours per mouse prior to euthanasia.

We used statistical methods to ensure that our datasets will have sufficient power for our analysis. However, as some of our work is basic science we had to estimate some of the parameters including duration of viable recording per animal, the variance in implant location, and the amount of data required for fitting statistical and deep learning models.

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 our anatomical targeting using up to 40 mice undergoing dy-I injections. Mice will undergo non-recovery anaesthesia and will not experience adverse effects.

We will also carry out internal assessments on the most successful and long-lasting animal implants to determine optimal parameters and possibly decrease the number of mice required across the duration of our study. Achieving stable and long-lasting neural implants and recordings are aligned to, and optimal, for our study goals and we will put effort into identifying how to obtain this.

We will run computational models of our datasets in parallel while collecting datasets. Evaluating the quality of our mouse models early should help us reduce or refine animal use along the way.

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 designed our research program to use the least sentient animal model and the lowest number of animals that are justified for our research goals. We selected mice because we require mammalian models to advance the neuroscience of agency knowledge relevant to human health and diseases such as schizophrenia and depression. Such diseases have specific genetic and neuroanatomical components present in most mammals and common between humans and mice [1] [2]. Additionally, there are existing studies that use mice to study the neuroscience of agency as well as studies implementing genetically altered (GA) mouse models of schizophrenia to study the effect of genetic

pathologies on behaviour and neural dynamics [3].

We will be housing mice in large, enriched home arena environments for the duration of the study.

We designed our study around socially housed animals and will seek to have a minimum of 2 mice and up to 4 mice per home arena. This will ensure higher quality of life for the subjects of our study and improve our outcomes.

All our mice have free access to food and drinking water which are monitored by trained staff. Additionally, mice will receive approved treats for performing optional and ad lib tasks in their home arenas.

In addition to standard animal inspection as per our protocol, we will implement active video monitoring of the cages using at least 4 video cameras per cage. The real time video data enables us to assess some of the health characteristics of mice without the need to handle the mice.

[1] Beauchamp A, Yee Y, Darwin BC, Raznahan A, Mars RB, Lerch JP. Whole-brain comparison of rodent and human brains using spatial transcriptomics. Elife. 2022 Nov 7;11:e79418. doi: 10.7554/eLife.79418. PMID: 36342372; PMCID: PMC9708081.

[2] Ruberte, J., Schofield, P.N., Sundberg, J.P. et al. Bridging mouse and human anatomies; a knowledge-based approach to comparative anatomy for disease model phenotyping. Mamm Genome 34, 389–407 (2023). https://doi.org/10.1007/s00335-023-10005-4.

[3] Rummell, B.P., Bikas, S., Babl, S.S. et al. Altered corollary discharge signaling in the auditory cortex of a mouse model of schizophrenia predisposition. Nature Communication 14, 7388 (2023). https://doi.org/10.1038/s41467-023-42964-2

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

We require mammals as we are studying the neural circuits underlying sense of agency in the mammalian brain. Additionally, we need live neural recordings as the dynamics and inter-area interactions cannot be replicated in-vitro or in computational models as they are largely unknown.

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

All mice will be monitored for adverse effects and undergo careful daily monitoring by trained staff using score sheets to monitor body weight and normal behaviours e.g. nest building. This will include increased monitoring throughout the day post surgery for 3 days and if necessary for more days, to ensure they recover from the surgery and are eating and drinking normally. Mice will be administered analgesics as required to ensure they are recovering as expected. Video monitoring which forms a central part of our study will also be checked daily as an additional method for monitoring.

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

We will follow best practice guidelines such as 'Animal Research: Reporting of In Vivo

Experiments' (ARRIVE) and 'Planning Research and Experimental Procedures on Animals: Recommendations for Excellence' (PREPARE) as far as applicable to our approach and research questions.

Moreover, we will stay informed by:

i) Using the National Centre for Replacement, Refinement and Reduction (NC3Rs) as a source of information and regular updates from the NIO.

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

Regular updates from The Laboratory Animal Science Association (LASA) and the National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs) are distributed within our institution. These organisations supply educational materials focused on improving animal welfare and minimising harm and we encourage lab members to study these and to participate in NC3Rs webinars.

We will also ensure that we follow the PREPARE guidelines to make sure our experiments are conducted in the most refined way possible.

6. The evolution of personality, plasticity and stress response in small fishes

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

behaviour, evolution, stress, fish, welfare

Animal types	Life stages
Zebra fish (Danio rerio)	Juvenile, Adult
Guppy	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 project will investigate the evolutionary origins and genetic basis of behavioural variation in fishes, with a specific focus on 'boldness'. Boldness is an aspect of animal personality that describes individual responses to risk in the environment, and that is now recognised as an integral part of the acute stress response.

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 aim of understanding the genetic basis of shy-bold variation in fishes is underpinned by both fundamental and applied questions.

First, despite decades of research, the evolutionary origin of variation in animal behaviour

continues to both fascinate and frustrate biologists. Understanding the behaviour of wild animals is important because behaviours form the first line of defense challenges in the environment (e.g., predators, climate change), and are critical to finding food and mates. This puts, behaviour on the ‘front-line’ of all interaction between individuals and their environment. Expressing the right behaviours in the right context and at the right time is critical for determining the health and evolutionary 'fitness' of animals. However, we still lack a comprehensive understanding of the evolutionary mechanisms that sometimes promote behavioural variation among individuals, populations and species, but - in other cases - can limit diversity and constrain divergence. Evolutionary models provide clear hypotheses and predictions, but these now need to be tested empirically. We will do this using shy-bold variation in guppies as an empirical case study.

Our second motive is more applied. Many fish species are kept in captivity for human use. This includes aquaculture species raised for food, ornamental species produced for the pet trade, and a growing number of fishes used in science (e.g. for biomedical studies). Globally, the number of fish held in captivity for human use if growing rapidly making it important to conduct research that will help us protect, and improve welfare. In captive populations of fish, welfare and health problems often arise when animals become 'chronically' stressed such that they are exposed to prolonged periods of elevated cortisol in their bodies. We now know that shy-bold behavioural variation is part of an integrated stress response in which behavioural and physiological processes have co-evolved to operate in tandem. We have shown that shy and bold individuals (and genotypes) differ in their stress physiology and, critically, can also differ systematically in welfare indicators when housed in captivity (e.g. growth, longevity). This implies that artificial selection on shy-bold behavioural traits could be an effective way to genetically improve 'chronic stress resistance' and welfare in captivity. To determine the feasibility, and effectiveness, of this selection strategy we need an improved understanding of the genetic basis of shy-bold variation, and the genetic basis of behaviour-performance relationships. To better explore this more applied aspect of the work and deliver proof of principle we will use a zebrafish model alongside our work on guppies, as this provides greater potential for translational impact.

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

The research will advance our basic knowledge of how and why behavioural variation evolves and persists in the wild. We find variation at 'multiple levels' of study - from genes, to individuals, and populations and need to study the relationships between these This is important because, for example patterns of behavioural similarity and/or difference between populations, depend on how natural selection has acted on behavioural differences among-individuals within each population in the past. Our fundamental research will therefore address the link between the process of evolutionary change within-populations (‘microevolution’) and the patterns of divergence that arise among-populations (‘macroevolution’). Although we will do this for the particular case of shy-bold behaviour, this link between micro- and macro-evolution is important to our understanding of all behaviours (and indeed other types of trait too). The fundamental component of our work using guppies will yield outputs in the form of publications in the peer-reviewed literature, conference presentations, and publicly archived data sets. At the same time, the parallel studies in zebrafish will provide insight into the feasibility and effectiveness of exploiting shy-bold variation as a tool to improve welfare in captivity. In addition to publications and presentations, a successful proof of concept study would allow us to generate additional outputs including practical tools and guidelines (e.g. what specific behavioural indicators to measure, how to design a low-cost screening and artificial selection strategy) for facility

managers and welfare scientists. We anticipate these outputs would be made freely available, no patents or commercial outputs from the research are currently expected.

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

Behavioural ecologists and evolutionary biologists - Immediate beneficiaries of the fundamental outputs will be biologists working from an evolutionary perspective to understand diversity in nature. Results will be particularly relevant to researchers in behaviour, animal 'personality', and stress biology. However, beyond the specific empirical focus our work also addresses general questions in evolutionary biology -specifically concerning the link between micro- and macro-evolution, and the importance (or not) of neutral trait evolution.

Welfare scientists and aquaculture research – The links between boldness (risk-taking behaviour) and physiological stress response that have emerged from our work so far also suggest potential downstream applications. This motivates the inclusion of the zebrafish component as a secondary model in which we will evaluate the practical utility of artificial selection on personality to achieve welfare gains in captivity. As such the study results relating to this species will primarily be of interest to researchers in welfare science and aquaculture genetics. Over the course of this project we expect the outputs to be academic. However, a successful proof of principle demonstration would set the stage for follow up projects to further develop practical tools and guidelines for zebrafish facility managers and animal technicians.

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

We will seek to publish the results of all work in the peer reviewed literature. This includes any 'null results' as documenting unsuccessful approaches and unsupported hypotheses is key to scientific advancement. We will use the ARRIVE guidelines to maximise the quality and reproducibility of our published work. In accordance with our ethical principles and funder requirements, we will continue to follow open science principles, publicly archiving data and code alongside our publications and favouring not-for-profit open access publication venues. We will also archive pre-prints to ensure universal access to the research. Results will also be disseminated at conferences and workshops focused on behaviour, evolution and (fish) welfare. The findings of this project will also be shared with the general public outside academia. We will do this by working with media, using social media outlets, and contributing to public engagement and outreach activities.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 2400
- Other fish:
    - Guppy: 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 guppies (*Poecilia reticulata*) and zebrafish (*Danio rerio*). Both species are

small, easy to manage and breed in captivity, and benefit from well-established and described husbandry procedures. For our purposes the guppy is particularly well suited because of its known ecology in relation to predation risk. Specifically, the guppy is found in rivers in Trinidad where barrier waterfalls restrict upward movement of large predators. This has created a natural experiment in which multiple rivers contain separate guppy populations that have evolved under high predation risk (below waterfalls) or low predation risk (above waterfalls). This 'risk gradient', coupled with the availability of recently wild-derived stocks from different sites and rivers makes them an ideal model to address the fundamental component of our project.

Although we could -in principle -also use guppies to address our applied objectives, for this part of the work the ecological drivers of past behavioural evolution are not directly relevant. Moreover, zebrafish are, by far, the most widely kept fish in scientific research, a sector that we believe is better positioned to rapidly adopt new welfare strategies than commercial aquaculture (i.e., food, ornamental fish production). Thus, we will use a zebrafish model here to maximise the potential for translational impacts.

We will use adults and juveniles of both species. Early embryos are not generally suitable for behavioural studies as planned and indeed cannot be studied in guppies. Guppies are a live-bearing species in which offspring are free-feeding and protected from birth.

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

Fish used will normally need to be individually identifiable as they will be subjected to repeated behavioural observations. In some cases, individuals may be uniquely identified from natural markings but more typically they will need to be tagged to allow for reliable identification. This will be done once they reach an appropriate size by injecting visible elastomer tags under the skin.

A typical individual will then be subject to non-invasive collection of data on behaviour and performance. Behavioural data will be collected by video tracking of fish introduced to experimental chambers for short periods (typically up to 10 or 15 minutes, sometimes up to 60 minutes on occasion). Data can then be collected on behaviour with fish exposed to standard assays (e.g. simple open field trials) and/or with presentation of additional cues (e.g. video presentation of a shoal of conspecifics, or a predator). Fish may be measured after behavioural observations so data on growth can be obtained. They may also be photographed to allow more detailed measurements to be made from images later. This process will be repeated on individuals, typically 4-6 times over a period of several weeks. We may also collect data on hormone levels using non-invasive methods in which hormone levels are assayed from the water. This is possible because hormones diffuse across the gills from blood into the water, meaning analysing water can tell us about what physiological processes are happening in the animal. Data on size/growth, maturation time, reproductive activity and longevity may also be collected using completely non-invasive methods already employed under standard husbandry.

Once fish are not required for further data collection or breeding they will be euthanized. In most cases this will be immediately following the completion of behavioural experiments. However, in some cases, fish will be kept alive for the duration of their lives to allow monitoring of growth, longevity and reproductive performance over longer time periods (e.g. a year). It is scientifically important to monitor performance over relatively extended periods representative of individual lifetimes, as evolutionary theory predicts that small genetic differences in health and performance in early life translate into larger, more

detectable effects over time. Euthanasia criteria (agreed with the named veterinary surgeon) will be in place to provide a humane endpoint for any fish that are diseased, injured or showing signs of health problems associated with old age.

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

The primary expected adverse effect is mild stress related to tagging fish under general anaesthesia and netting them to move them into experimental tanks. We expect this to be short-lived as fish typically exhibit normal behaviour within several minutes of being tagged and netted. Mortality (<1%) can occur in rare cases with a fish failing to recover properly from anaesthesia.

Some limited bruising or bleeding under the skin at the site of tagging can be encountered (<15%) but such fish exhibit normal behaviour and recover fully within a few days. The non-invasive behavioural and physiological data collection is considered very safe - for instance we have encountered no adverse effects directly attributed to our assays in >15,000 behavioural trials of guppies. Nonetheless, fish will experience some mild, transient distress during capture and handling for collection of length/weight data, and when being transferred to experimental tanks for behavioural data collection. Moreover, because we will require repeat observations of behaviour from individuals, we will limit the total number and frequency of repeats per individuals to minimise any risk of adverse effects from cumulative stress.

A subset of the zebrafish used will be deliberately subjected to a 'chronic stress assay' which is intended to mimic levels of husbandry induced stress that are likely to be encountered in poorly run zebrafish facilities. These fish may show moderate adverse effects in some cases. These will occur principally in the form of reduced growth rates (or weight loss) during the experimental assay (typically expected to last up to 21 days). Fish health is monitored daily using a set of criteria that assess demeanour, appearance, behaviour, skin changes and buoyancy. Additional signs of chronic stress include loss of appetite, abnormal behaviour (gasping at the surface or lying at the bottom of the tank) and change in colouration.

Beyond rare mortality associated with tagging, we do not expect any elevated mortality risk of fish to occur as a direct result of licensed procedures. However, since our planned experiments are long- term, with some fish being monitored for months or even years to collect data on growth and longevity, it is inevitable that there will be background mortalities (i.e. some fish may be found dead or culled due to poor health). This mortality will be no higher than the general background rate of mortality in the facility.

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 fish used in licensed procedures will be under a single protocol with a moderate prospective severity limit. We expect 100% of guppies and 87.5% of zebrafish to experience only mild severity as they will not be subject to the chronic stress assay (an optional protocol step). We expect the remaining 12.5% of zebrafish (an estimated 300 individuals) will experience this assay and may 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?

The aim of the project is to understand the evolution of animal behaviour, and specifically to investigate the genetics of a phenomenon known as 'animal personality' in fish. We have well developed theoretical models to predict when, and how, behaviours will evolve under selection. However, empirical data are needed to test our theory. This means we must collect behavioural data on live animals (in this case fish).

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

We cannot study animal behaviour in plants or prokaryotes. We did consider less sentient non- protected animals.

Why were they not suitable?

For investigating general behavioural genetic phenomena that are not taxon-specific we are increasingly using non-protected invertebrate models (e.g., sea anemones, cherry shrimp). However, for this project fish must be used since (i) we seek to build on established links between animal personality and the stress response (which is highly conserved across vertebrates, but not invertebrates), (ii) the fundamental components of the work are taxon-specific (i.e. we are using guppies precisely because our hypotheses are dependent on their well-described natural ecology), and
(iii) the applied components of the work are also taxon specific (i.e. we aim to test whether personality variation can be harnessed as a practical tool to improve welfare in captive fishes).

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 estimate is based on our published data and pilot data from previous experiments. We have previous experience in using guppies and can therefore accurately estimate the number of animals needed in each population to obtain statistically valid results for anticipated effect sizes. As we generate more results, we will continually assess sample sizes to determine if numbers can be reduced from those currently proposed. Specifically, we will also use simulation-based methods to explore whether further reductions in sample

sizes are possible without compromising insights from subsequent data analysis.

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

We have based our sample size on previous experiments using similar protocols and on simulation- based approaches that allow us to investigate statistical power for the planned genetic analyses of behaviour. These have proven successful at enabling us to detect biologically relevant effect sizes (e.g. proportion of behavioural variation due to genes in a population). Our proposed experiments include multiple behavioural observations per individual precisely because this allows for more precise characterisation of individual personality which, in turn permits robust conclusions to be drawn from smaller sample sizes. More observations per individual raises a risk of cumulative adverse effects, however, so in practice all our experiments require consideration of a trade-off between reduction (limiting the total number of animals) and refinement (limiting the number of observations, and so total experimental experience, per animal). We also plan to use statistical modelling methods from quantitative genetics and evolutionary biology (e.g. mixed models) that are more powerful than classical statistical methods for the complex data sets we will produce. We have also consulted online resources (e.g., PREPARE guidelines, NC3Rs) to ensure our plans are in line with best practice.

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 used previously collected data and simulation-based approaches to determine suitable sample sizes for the planned work. Wherever possible will also use videos and/or chemical cues of conspecific fish and predators (e.g. cichlids) in our behavioural assays of shy/bold personality. This avoids large numbers of additional animals being 'used' (albeit not for regulated procedures) simply to provide environmental cues to elicit behaviour. We will use automated tracking software to measure fish behaviour, which greatly reduces measurement error and so increases statistical power for a given sample size (as well as eliminating subjectivity and risk of observer bias).

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.

Choice of animal models
The fish models to be used are exceptionally robust and easy to care for and breed under lab conditions. General welfare will be ensured by maintaining housing conditions and husbandry standards (e.g. daily inspections, frequent water changes, a robust program of water quality testing) that meet or exceed all HO requirements.

We will use **guppies** as our primary model to address the fundamental scientific

objectives. The Trinidadian guppy (*Poecilia reticulata*) has been chosen for several reasons. First, it is easy to breed and maintain under laboratory conditions. Secondly, it has a well described ecology, and the role of predation risk in driving evolution has been extensively documented. Third, we already have access to recently wild-derived captive colonies from multiple rivers in Trinidad. These populations are from sites with known differences in predation regime. The use of a recently wild derived model is essential for our study - we need access to multiple outbred populations from different predation regimes with no history of artificial selection.

We will use **zebrafish** (*Danio rerio*) as a secondary model in which to explore possible application of personality-assays as a tool to improve welfare outcomes in captive fishes. This species is also an appropriate model given its small size, ease of breeding, and well-established husbandry requirements. However, they are additionally the most relevant species for our applied aims. They are by far the most widely housed fish in scientific research and, despite well-established husbandry protocols, stress-related welfare problems persist in captivity.

Choice of methods
Tagging - We will primarily use visible elastomer tags to identify fish as the adverse effects of the procedure are short-lived. Alternative options like individually housing fish to keep track of identities are not feasible for large numbers and are likely to create more adverse effects and may lead to chronic stress outcomes. We may occasionally use ultra-small passive integrated transponder (PIT) tags (e.g., 500x500x100µm injected by pre-loaded disposable needle) if we deem this a refinement in a specific experiment. This tagging system offers large numbers of unique identifiers which can facilitate tracking of individuals housed in larger groups without confounding individual identification. However, in most cases the benefits - relative to elastomer tagging - will be more than offset by the need to capture and handle a fish to identify it from PIT tag. In contrast, elastomer tags are visible on fish in home tanks without any need for capture.

Assays of behaviour and physiology - all methods are entirely non-invasive and there are no alternatives of lesser severity. All assays require some handling and transfer from home tanks to arenas or other chambers. As with all standard husbandry practices we will minimise stress caused using gentle netting and handling by experienced trained researchers. Behaviour will be assayed using video tracking of behaviour in experimental arenas and (less commonly) by 'indirect' human observation (e.g. in real time but via a camera to avoid disturbance by the experimentor). We will use a battery of standard tests common to zoology and comparative psychology to assay aspects of personality. These include open field trials, emergence trials, social interaction (e.g. shoaling) and predator response trials. We will often combine multiple cues (and so assay types) into a single trial allowing us to get richer, multivariate data on individuals with fewer capture and handling events. All behavioural assays are designed to measure the normal behavioural repertoire of the fish. In testing predator responses we will primarily use video stimuli but may on occasion use live predators (e.g. to validate that models elicit equivalent responses), with focal fish always physically isolated and provided with a refuge allowing escape from visual contact. We may also simulate predation risk by net chase or similar disturbance of short duration (e.g., 30 second chase with net), and/or by exposure to waterborne cues (e.g. conspecific alarm cues, water that has previously housed predators). These stimuli are widely used to mimic predation for ecological studies but are also a relevant representation of experience in captivity (i.e. handling is 'predation by humans'). Moreover, they are very safe. Since 2012 we have conducted over 10,000 assays of personality on small fish models, including but not limited to tests of response to predation risk, without detectable

adverse effects. To assay stress physiology we will use waterborne endocrine assays that provide substantial refinement over traditional blood sampling methods (or lethal sampling)

Testing performance under chronic stress - will be needed for a subset of zebrafish. Although we must deliberately subject some individuals to sub-optimal conditions, the planned protocol is as refined as possible. We will use weight loss (or reduced weight gain) as the least severe endpoint available to us that indicates an adverse effect of chronic stress. To test performance under chronic stress, fish will be exposed to an unpredictable sequence of of specific events (net chases, reduction in water level, exposure to transient temperature increases/drops, high volume water changes, short term crowding/isolation) that been selected since they are expected to have only mild adverse effects individually. All stimuli are representative of stressors that can and do occur routinely through (poor) husbandry practices in captive populations. The variety of stimuli to be used within the assay is necessary since it is expected that chronic stress effects arise in part from a lack of habituation (reduced sensitivity to stress stimuli). In turn habituation is known to occur more readily and rapidly if stress stimuli applied are 'stereotypic' (i.e. an identical stressor stimulus is applied rapidly).

Monitoring long-term performance of fish - to determine whether individual behavioural profiles (or personalities) predict longer term performance fish some will be kept on license for the duration of their lives . We anticipate this may be for up to 2-3 years in some individual cases. The reason for this is that we are trying to characterize variation in performance, which in the context is the ability to live a long healthy life, and to determine behaviours that predict which individuals carry the good genes. To quantify variation in performance it is important that we do not mask it by impose an arbitrary age of death on healthy individuals and this is the reason for the long-term performance monitoring. The data collection for this is non-invasive and involved no further steps that cross-threshold. It can mostly be done without handling fish. However, to collect data on growth and mass some handling will be needed. We will take steps to minimise the impact of this, for example by obtaining measures at the same time fish are being handled for transfer to behavioural or physiological assays. Where possible we will take length measurements from photos (to reduce handling and emersion time), and weigh fish in water by placing them in a beaker of water placed on a tared mass balance. Where longer term monitoring is required, fish will be checked daily for signs of senescence (e.g. loss of appetite, weight loss , listless swimming performance, increased susceptibility to infectious disease and, on occasion, development of spinal deformities in very old fish). In practice humane endpoints agreed with NVS for euthanasia are in place in the lab such that fish showing any signs of senescence are euthanised before welfare is compromised. Assays of longevity will therefore be refined by using age at euthanasia as a proxy for longevity.

Euthanasia - Finally, we are requesting authority for non-Schedule 1 killing in which we may use the ice slurry method as a refinement for euthanasia. This causes faster death and has been shown to be less aversive than other commonly used methods. This involves introducing a fish to an ice bath, where the ice is away from the animal, for at least 10 minutes. Death is confirmed by destruction of the brain.

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

There are no non-sentient alternatives for empirically addressing the research aims. This is a study of animal behaviour: it is not possible to study behaviour of animals under terminal anaesthesia. Fish must be used since (i) we seek to build on established links

between animal personality and the stress response (which is highly conserved across vertebrates, but not across less-sentient invertebrates), (ii) the fundamental components of the work are taxon-specific (i.e. we are using guppies precisely because our hypotheses are dependent on their well-described natural ecology, and (iii) the applied components of the work are also taxon specific (i.e. we aim to test whether personality variation can be harnessed as a practical tool to improve welfare in captive fishes). Note that the primary model to be used is the guppy, a livebearing species that is free-feeding from birth and does not have a less sentient lifestage.

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

As described above, we believe the methods to be applied for data collection are already refined in the sense of minimising invasive procedures and expected adverse effects. However, additional refinement comes from careful monitoring of individuals pre- and post-procedure. This ensures that only visibly healthy animals enter experiments, and that systems are in place to detect any unexpected adverse effects that do arise. We also manage experimental timelines and social environments carefully to avoid cumulative stressors arising. For example, we preferentially tag fish early in the week to ensure any rare adverse effects of anaesthesia do not coincide with a reduced level of staff observation over the weekend. Following tagging, fish are typically returned to the same social groups, or a sub-set thereof, to avoid novel social stressors (e.g. from disrupted dominance hierarchies) and typically observed for a minimum of 7 days before entering behavioural testing.

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

For designing and implementing experiments we follow the ASAB Guidelines for the ethical treatment of nonhuman animals in behavioural research and teaching. In outputs describing this work we also follow the ARRIVE guidelines.

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

Information about 3Rs advances is disseminated to all license holders and named persons via our institution's ethics team. Additionally, in my own ethics governance roles I continue to seek, and receive information directly from external organisations (NC3Rs, RSPCA), and through academic networks (e.g. I am part of the UK-wide Animal Welfare Research Network, AWRN), as well as via engaging with the peer reviewed literature on fish welfare (as a reader, author and reviewer).

Locally, all users of the Animal Facility meet weekly with NACWOs to discuss ongoing work and evaluate any possible refinements to protocols as well as to the facility’s standard operating procedures covering routine fish husbandry methods.

7. Epigenomic regulation of cardiovascular phenotypes and its impact on disease susceptibility

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

Basic research, Cardiovascular, Gene regulation, Epigenetics, Cardiac hypertrophy

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 project aims at addressing the mechanisms controlling where and when genes are expressed in the cardiovascular system, with a focus on heart tissue. Our experiments will characterise the role of these processes during development and differentiation, and how they impact organ-level functions and human susceptibility to cardiovascular disease. We will also use specific models of cardiac stress and remodelling, focusing on the development of a hypertrophic, enlarged heart, to test the role of specific mechanisms in mediating susceptibility to 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?

Cardiovascular diseases typically present in the older population and with variable penetrance and severity. Such heterogeneity is thought to be influenced by both genetic and environmental factors, with genomic studies in human populations implicating DNA regions outside genes in interindividual susceptibility to disease. This observation highlights the relevance of studying the impact of gene regulation in development, adult organ function and its progression to disease.

Moreover, cardiovascular diseases such as cardiac arrhythmias, cardiomyopathies and heart failure often occur in the context of chronic stress, such as that caused by hypertension, aortic stenosis or myocardial ischaemia. How particular stressors such as hypertension or mechanical overload influence susceptibility, onset and penetrance of cardiovascular disease is an active area of investigation with the potential to inform novel prognostic and therapeutic approaches in the long term. Our choice of cardiac hypertrophy models is motivated both by the well-known role of hypertrophic processes in mediating diseases of the heart muscle, and by the high prevalence and societal burden these diseases represent.

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

A key output expected from this project are novel, fundamental insights into the mechanisms of gene regulation in the cardiovascular system. The project is expected to contribute to an understanding of how these regulatory mechanisms can influence the development of disease, and how they may be associated with susceptibility to specific forms of heart disease - such as impaired heart muscle function. These outputs will be materialised in scientific publications, including conference presentations and articles in specialised journals.

Our work will also generate reference datasets made available to the research community, which can and have often been used to address complementary questions by other research groups. The information provided by our work is of potential use to scientists working in pre-clinical research. For example, we will provide knowledge that could improve the ability to link genetic differences between individuals with disease susceptibility. We will also utilise in vitro human models and clinical samples, and outputs generated in these resources will further inform molecular disease mechanisms and potential prognostic or diagnostic candidates. However, any pre-clinical benefits are beyond the scope of this immediate project.

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

In the short term, early sharing of research outputs, for instance in conference presentations, will maximise dissemination benefits to the scientific community. These include sharing of knowledge and generation of new hypotheses, but will also contribute to minimise unnecessary duplication of equivalent animal experiments by research groups with similar interests.

In the medium term, project outputs will benefit the scientific community through peer-reviewed publications and research datasets. The first will contribute to the generation of new knowledge and open further research avenues, including in the pre-clinical research area. The second is an important benefit of the types of data we will generate in the project, which will be made publicly available through specialised repositories. Such extended sharing of research datasets can allow interested researchers to further benefit

from our work, for instance by addressing complementary research questions or integrating our data with existing or newly generated datasets.

Following completion of the project and in the longer term, our findings can contribute to pre-clinical research into the epigenomic contributions to cardiovascular disease. Our outputs from this project are expected to associate specific epigenomic regions with disease susceptibility. Such candidates could be evaluated in a pre-clinical screening setting, for instance to develop novel prognostic tools for early disease prevention.

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

We will use a number of approaches to maximise outputs from this work. First, we will share research outputs as early as possible, for instance through conference presentations, scientific seminars and pre-print publications. Second, we will collaborate locally and internationally with groups sharing similar interests, to ensure our outputs can be useful to other researchers, and be expanded through collaboration by addressing complementary questions. Third, we will share datasets and analyses with the scientific community as early as possible, and upon publication of findings at the latest. This will ensure maximal research outputs from our work. Lastly, we will publish our findings in open access journals and pre-print servers to ensure wide accessibility to scientists and other stakeholders such as research funders and regulatory agencies.

Species and numbers of animals expected to be used

- Mice: 1150

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.

Laboratory mice are an invaluable research model with unparalleled availability of genetically-modified strains. We also have substantial prior experience in mouse models of cardiovascular function.

Our choice of life stages is informed by the scientific questions we will address. We use juvenile or adult mice to test the impact of specific alterations during development and differentiation. To study the impact of gene regulation mechanisms on cardiovascular phenotypes and disease predisposition, we will use adult mice as they more closely recapitulate the physiology of adult humans in which cardiovascular disease occurs.
Typically, what will be done to an animal used in your project?

The majority animals will be used for breeding and maintenance of research colonies, with only around twenty percent of mice in the project being used in experiments associated with adverse effects.

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

Many of our studies use tissue samples collected from mice which have no expected

adverse effects and which have been killed by a humane procedure. Some studies use mice carrying mutations which may cause impaired tissue function or growth retardation. For these mice, we control mutation dosage to restrict adverse effects to a mild severity, and closely monitor mice for any unexpected defects during early post-natal life.

We also use mice treated with specific substances or subjected to cardiac surgery to modify cardiovascular function, and then allowed to develop impaired heart function. Some of these mice are also subjected to imaging studies to measure changes in cardiovascular physiology. During this work, we conduct studies at early time points and the mildest possible severity, thereby minimising the adverse effects animals may experience. We also provide best care to prevent pain (with anaesthesia and analgesia) and avoid animal suffering. However, adverse effects such as development of an enlarged heart, an irregular heart beating, abnormal weight loss, reduced mobility or altered respiratory rate are likely to be unavoidable to meet our scientific objectives. Some animals may experience rare post-operative complications which are not part of our scientific goals such as neurological, metabolic, or digestive dysfunction, infections or build-up of abdominal fluid. These mice are immediately euthanised to avoid suffering.

We intend to humanely kill experimental mice before or at the onset of moderate signs of illness. Because animals will be intensively monitored, we expect to be able to intervene in this way to ensure no progression beyond mild to moderate severity. Experimental animals are killed by a humane method at the end of the study.

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 mice in our project, such as those used for breeding of genetically-altered strains, we expect a mild severity as they will not be subjected to procedures with significant adverse effects. For these mice, we control mutation dosage, and closely monitor mice for any unexpected defects during early post-natal life.

Mice used for surgical interventions or imaging have an expected moderate severity, as these procedures are associated with discomfort and clinical signs. During this work, we conduct studies at early time points and the mildest possible severity, thereby minimising the adverse effects animals may experience. We also provide appropriate welfare monitoring and care to prevent pain (with anaesthesia and analgesia) and avoid animal suffering.

We intend to humanely kill experimental mice before or at the onset of persistent moderate signs of illness. Because animals will be intensively monitored, we expect to be able to intervene to avoid any progression beyond moderate severity. Experimental animals are killed by a humane method at the end of the study.

Overall, we estimate around 88% of mice in our studies will experience a mild severity, and expect up to 12% of animals to experience moderate severity. These estimates take into account animals' lifetime experience, such as those of mice used in breeding and subsequently undergoing surgical interventions.

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?

Post-mortem tissue samples from animals are essential for our studies to understand normal tissue- specific gene regulation, such as that operating in the heart. For our experiments investigating how, where and when genes are expressed during cardiac stress, animal samples are crucial for similar reasons.

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

We use *in vitro* experiments (i.e. cultured cells in a dish) extensively to investigate cardiac differentiation and the development of an enlarged heart muscle. In our previous work, we have established *in vitro* human models derived from pluripotent stem cells that mimic some physiological aspects of the development of an enlarged heart muscle cell. Whenever possible, we also access human tissue samples for ex vivo investigations from local tissue banks in our hospital environment. Experiments in these non-animal systems allow us to test initial hypothesis using in vitro models, and to investigate the potential human relevance of our work in patient samples. Whenever possible, we also use available data generated by other groups or accessible in public research databases to answer our research questions.

Why were they not suitable?

Non-animal approaches such as cell culture in a dish are of limited use for our research, since the gene regulation found in cultured cells drifts substantially from that in the original tissue from which the cells were derived. Tissue biology is profoundly influenced by its environment within the body and the interaction between different types of cells, and in vitro methods in a dish are unable to faithfully mimic this complex interplay.

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?

Estimates are based on the numbers of mice needed to maintain genetically altered lines and to generate experimental animals, and on the predicted number of genetically altered lines needed on this project licence. For animals used in experimental procedures, estimates take into account our scientific objectives, the types of data we generate and the

specific research models. In particular, we consider experimental variability for the readouts we measure in experimental animals, as well as the technical and biological variability associated to surgical or imaging procedures in previous studies. We also assign additional animals for pilot studies with small numbers, to establish time-points for sample collection and dosing regimes for substance administration.

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

Animal numbers are minimised by designing husbandry and welfare monitoring strategies to produce experimental animals as efficiently as possible, and by the use of statistical analyses to determine the minimum number of animals required without compromising the scientific aims of the experiments. We also use pilot experiments with small numbers of animals to validate experimental conditions and best refinements before conducting larger studies, and ensure we maximise the utility of results by careful reporting of experimental conditions and conclusions.

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

We implement efficient breeding strategies and pilot studies with small numbers of animals. We collect multiple samples from experimental and control animals to form a tissue bank for future ex vivo experiments. If possible, we use tissue from this collection rather than use new animals. Careful banking of tissues also allows us to use samples from the same animal in multiple studies, and share tissue samples with other research groups addressing complementary 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.

We will use animal models of an enlarged heart and impaired cardiac function, and imaging or electrophysiology methods to measure heart function over time. Our choice of whole-body and organ- specific models of cardiovascular stress is based on the experimental needs to introduce perturbations in tissue function, and to use organ-specific models of clinical relevance (such as models of an enlarged heart, a condition which often associates with heart failure in humans).

Appropriate health monitoring protocols, such as increased close monitoring, are implemented for mice expected to have adverse effects, for example caused by surgical interventions. Aseptic surgery and suitable pain killers (i.e. anaesthetic and analgesic regimes) are used to minimise suffering. Animals are closely monitored so we can intervene before or at the onset of clinical signs, or are killed by a humane method at the end of the study.

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

The mouse is the experimental animal of choice for this project. The availability of extensive genetic resources in mice, such as many well-characterised and readily available genetic alterations, are crucial for our studies of cardiovascular gene regulation. There is reliable technology to genetically manipulate mice and an extensive inventory of readily available genetically modified mice. This resource allows us to assess changes in gene regulation (e.g. in the heart) caused by altering specific components of gene expression control.

Less sentient animals such as fish are much more distant in their cardiovascular anatomy and physiology from humans, and are therefore unsuitable to address our scientific objectives. Similarly, some of our experiments require assessment of alterations in cardiovascular physiology and heart function over time, which is not possible in terminally anaesthetised animals.

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

We will continuously review the monitoring regimes and humane endpoints in our procedures as our experiments progress, and implement refinement measures as appropriate. These can include increased monitoring should we observe clinical signs with a higher frequency than anticipated, or refinements to pain management and post-operative care following surgical procedures.

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

We will follow general regulatory guidance, such as PREPARE guidelines for planning animal research and testing (Smith et al. 2018) and Rinwa et al. “3R-Refinement principles: elevating rodent well-being and research quality” (2024). We will also keep abreast with developments in refinement of the specific animal models we use.

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

We will stay abreast of advances in the 3Rs through established seminar series in our local environment (such as annual NC3Rs symposiums and research seminars organised by our animal services unit), online resources and the development of grant applications over the course of the project (including in areas aligned with the 3Rs). These will allow us to implement any advances effectively, for instance by incorporating improved in vitro models into our research, or improved refinement of animal experiments.

8. Delta granules from platelets as source of pathological calcification

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Pathological calcification, Cardiac diseases, Cardiac calcification, Calcium

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 project aims to determine whether calcified spherical particles (associated with cardiovascular disease) present in the vascular system originate from delta granules that exist within platelets and can be released by 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 cardiovascular system is one of the most common sites of pathological calcification (formation of minerals caused by a disease). Indeed, cardiovascular calcification is a widespread phenomenon involved in several cardiac diseases (as aortic valve stenosis, rheumatic fever and atherosclerosis) that, taken together, contribute to close to more than 18 million deaths worldwide per year. The only treatment currently available for cardiovascular calcification is surgery. As opposed to most cardiovascular diseases, cardiovascular calcification is not associated with any lifestyle or diet, and therefore cannot be prevented by changes in individual behaviour.

A few years ago, we discovered that cardiovascular calcification is formed from calcified spherical particles. We also discovered that these same spheres are also the first minerals to appear in calcific diseases and that the mineralised spheres that appear in different cardiac diseases are the same.

In the last couple of decades, cardiovascular diseases such as atherosclerosis, rheumatic fever, and aortic valve stenosis have all been connected to blood platelets.

Platelets are a component of blood, but platelet vesicles can also be found in circulation. One of the components present in the platelets and also free in the blood is called delta granule, it has a size of around 100 nm, and has high concentrations of calcium and phosphorus (the same elements that form the minerals present in cardiovascular disease).

Since cardiovascular calcification is formed from calcified spheres with similar size, composition, and morphology to delta granules, and having considered the strong connections between platelets and cardiovascular disease, we propose the following hypothesis:

The calcified spheres, present in all stages of the cardiac disease, are indeed delta granules from platelets that have mineralized.

Following this hypothesis, in the last few years, we confirmed the presence of platelet proteins in the calcified spheres, confirmed that these calcified spheres present the same composition, shape, and size as delta granules from platelets, and found that these calcified spheres are present in every animal that have platelets. At the moment, we are running cardiac cell cultures with platelet vesicles to reproduce the calcification found in vivo.

Recently we found that there is an animal model (mouse with a mutation in HPS genes) that reproduces the disease called Hermansky-Pudlak syndrome. In this syndrome, platelets do not have any delta granules. Interestingly, when this mutation is combined with another mutation that makes animals prone to heart attacks, researchers have shown that it is not possible to make these animals develop a heart attack, showing that mutations on the HPS genes indeed protects the animal from cardiac diseases.

Therefore, in this project, our intention is to use mouse models to show that the animal model for the Hermansky-Pudlak syndrome (HPS3-/-), also does not present any cardiovascular calcification, when compared to normal mice and mice prone to heart attacks. If the animals (HPS3-/-) that do not have delta granules do not produce any cardiovascular calcification, we will have confirmed that cardiovascular calcification originates from platelets, and more specifically, from their delta granules.

To test our hypothesis, we will have animals with mutation (HPS3-/-), animals prone to calcification (APOE-/-), and animals without any mutations eating a high-fat diet and a normal diet. In normal circumstances, calcification will develop naturally in all animals but the progression of cardiovascular disease would increase with a high-fat diet.

The procedure in this project would be a single collection of a small amount of blood from the mice (by tail vein collection), to confirm the characteristics of their platelets. We would then leave the animals to age, each group with their specific diet (high fat or normal diet).

At one year and one year and a half, animals will be non-recovery anesthetised, then imaged by CT- scan, and then euthanised using Schedule I methods so that their aortas can be harvested. Harvested aortas will be characterized by fluorescence and electron microscopy, and biochemical methods to search for calcification.

With this project, we hope to suggest a new mechanism for the origins of calcification in the vascular system including a new, early, and hitherto unaccounted key event in the process of cardiovascular calcification. This new mechanism, along with a better understanding of the early stages of cardiovascular calcification, could open the path for the development of pharmacological prevention and treatment solutions for several cardiac diseases.

By identifying the disease-specific origin of calcification, specifically the platelet delta granules, we may be able to develop novel approaches to slow or halt the progression of cardiovascular calcification across various diseases. This research is also the step stone toward discovering potential biomarkers in platelets or platelet delta granules for early detection of calcification, helping to prevent cardiovascular diseases before they reach advanced stages.

In the long term, our goal is to leverage these insights, based on confirming the role of platelets in cardiovascular calcification, to inform the development of new diagnostic and therapeutic methods for calcification-related cardiovascular diseases. With cardiovascular disease contributing to over 17 million deaths annually and current treatments for calcification limited to surgical intervention, this project has the potential to lead to life-saving preventive and therapeutic strategies, fundamentally shifting the approach to managing these conditions.

Finally, this project has the potential to generate broader scientific insights. The process of mineral formation in the body is not limited to cardiovascular tissues—similar processes occur in other diseases, such as cancer, Alzheimer’s disease, kidney stones, and osteoarthritis. Therefore, the outcomes of this project could have far-reaching implications across multiple fields of medicine.

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

With this project, we hope to better understand the origins of cardiovascular calcification, a phenomenon that is associated with most cardiac diseases.

Understanding the origins of cardiovascular calcification, which cells are responsible for calcification, and how these cells create calcification is the necessary first step toward the creation of prevention methods against cardiovascular disease.

The results of this project will be submitted to scientific publications and disseminated via presentations at scientific conferences.

In the long term, our goal is confirming the role of platelets in cardiovascular calcification, to inform the development of new diagnostic and therapeutic methods for calcification-related cardiovascular diseases.

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

If we confirm the platelets or platelet vesicles are responsible for cardiovascular

calcification, we also will have an indication of the mechanism responsible for cardiovascular calcification, which will therefore make it possible to identify pharmacological targets to prevent the disease. Therefore, the first beneficiary of the outputs of this project will be drug development researchers, who will have a new road map to create pharmaceuticals that could prevent cardiovascular calcification.

In the long run, the beneficiaries of this project will be patients, not only those that suffer from cardiovascular calcification but also patients that suffer from other diseases that also present calcification. This project could also provide new information about calcification that occurs in the context of several other diseases.

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

We plan to disseminate all the results (unsuccessful outcomes as well and successful) through scientific publications. We will also target publication and presentations of outputs through conferences, meetings, and workshops.

All publications will be open-access and feature raw data as Supplementary Information as much as possible. All raw data will be available upon request to other researchers at any time.

Publications will initially be submitted to pre-print services (as bioRxiv or medRxiv). In this way, we guarantee that any outputs of this project, even if not accepted for publication, will still be public knowledge.

Finally, the animal model used in this project could also be used to understand other pathological calcification in the future. Through collaboration, we will share the data with researchers that work on pathological calcification in different diseases.

Species and numbers of animals expected to be used

- Mice: Total animals: 130

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 has a specific hypothesis: iCardiovascular calcification (that is formed by calcified spherical particles) is originated from the delta granules from platelets.

Therefore, to confirm or refute this hypothesis, an animal that does not have the delta granules is needed, and testing would look at whether these animals have (or not) calcified spherical particles in their tissues. Therefore, for this project we need an animal model that does not present the delta granules in their platelets, that is the mouse with a mutation in gene HPS.

For the life stages, since cardiovascular calcification changes and increases with age, to confirm the origins of cardiovascular calcification we need to have animals at old ages. We are looking to simulate, in this way, what is observed in humans for cardiovascular

calcification.

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

Animals will be bred Initially, we will breed three strains of animal models: APOE-/-, and HPS3-/-.

The animal models plus the wild type will be fed either a regular diet or a high-caloric diet over their lifetime.

The collection of small amounts of blood from mice would be an intervention of mild severity.

At the end of the experiments (first time point at 12 and second time point 18 months), the mice will be humanely killed.

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

Some groups of mice that are used in our study will be genetically modified, and they may exhibit a higher risk of blood coagulation problems, with coagulation times approximately 7 times longer compared to wild-type animals. In addition to their increased susceptibility to bleeding due to the mutation, these animals may also have an elevated likelihood of developing fibrotic restrictive lung disease (decreasing the capacity of the lungs) at late age, and potentially developing tumours as they age (with no higher incidence than what is observed on the wild type animals, around 1% of animals presenting tumours at the age of 18 months).

Additionally, those subjected to a high-caloric diet may experience the potential onset of obesity, have high levels of insulin and sugar in their blood, and have high blood pressure. Also, the animals will undergo significant increases in both overall body weight and localized adiposity.

There is also the risk that some animals may succumb to diabetes mellitus, metabolic syndrome, and vascular dysfunction as a consequence of these dietary conditions.

Moreover, because of the ageing, animals can experience:

Decreased Mobility: Aging animals may experience a gradual decline in mobility and activity levels. This can manifest as reduced agility, joint stiffness, or slower movements. These changes are typically mild and do not cause lasting harm.

Reduced Sensory Function: Aging can lead to sensory changes, such as diminished vision or hearing. While these changes can affect an animal's perception of its environment, they are generally considered part of the natural aging process and are not severe or lasting adverse effects.

Metabolic Changes: Aging can result in alterations in metabolism, potentially leading to weight changes or changes in energy levels. These changes are typically gradual and manageable, and they do not cause severe or lasting harm.

Increased Susceptibility to Age-Related Diseases: Aging animals may become more

susceptible to certain age-related diseases, such as osteoarthritis or age-related cognitive decline. While these conditions can impact an animal's quality of life, they are not caused by the aging process itself but rather by age-related changes in physiology.

On the project, we will also collect blood from the animals once .

At the end of the experiments (12 and 18 months), the animals will be humanely killed by Schedule 1 methods.

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)?

Breeding: We will breed and maintain genetically altered mice, and part of the animals will be aged and fed a high-fat diet.

We anticipate that breeding and maintaining genetically altered mice may cause moderate pain to the animals. This will involve around 130 animals (Apoe-/-; HPS3-/-), thus affecting 100% of these animals. The severity of this procedure is moderate.

Maintenance: The high-fat diet will be administered to half of the animals and may also cause moderate pain. Consequently, this will impact 50% of the animals. The severity of this procedure is moderate.

Aging: Ageing will be applied to all animals and could result in moderate pain. Therefore, this procedure will affect 100% of the animals in the study. The severity of this procedure is moderate.

The animals selected are genetically modified animals that could have problems with blood coagulation, having around 7 times longer bleeding times compared with the wild type. Therefore, special attention needs to be paid to any injury that can happen inside their cages. In addition to their increased susceptibility to bleeding due to the mutation, these animals may also face a higher likelihood of developing fibrotic restrictive lung disease (decreasing the capacity of the lungs) and potentially developing tumours as they age with no higher incidence than what is observed on the wild type animals (around 1% of animals presenting tumours at the age of 18 months). Therefore, could be expected a moderate level of severity.

From blood collection, (this will be collected from the animals only once) we expect mild severity to be caused to animals.

At the end of all the experiments, the animals will be humanely killed by Schedule 1 methods.

The overall severity, taking into account all procedures, is that 100% of the animals will experience a moderate severity level. This assessment was determined considering that no procedure exceeds moderate severity and accounting for all mitigating factors.

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 our project, we are already using human tissue and cell cultures to study the possibility of cardiovascular calcification having originated from platelets. However, to finally confirm without a doubt that this calcification as a whole and with all the characteristics observed in the tissue has their origins in the delta granules from platelets, we need to have an animal model where platelets are lacking the delta granules. Therefore, we need to use animals and cannot replace the animal model suggested here. Cell cultures cannot reproduce the whole tissue complexity, and neither can they reproduce the age-related accumulation of calcification observed.

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

We already considered cell cultures and, indeed, we are already using cell cultures and looking at human tissue samples At the cell culture we are looking the effect of platelet dense granules in the calcification of the culture of vascular cells and on tissue we are looking at for platelet marker and checking how they correlate with the calcified spherical particles. Still, we cannot have a look at the full system without an animal model. This is all the more critical for this project because our hypothesis is that cardiovascular calcification comes from the delta granules from platelets and, unfortunately, platelets cannot be cultured. Moreover, it is not possible to reproduce in vitro the interaction between platelets and real tissues.

Why were they not suitable?
The cell cultures that we are studying can only reproduce the interaction between one kind of cell and some of the vesicles from platelets. However, our hypothesis is that the vesicles from platelets get stuck on the tissue and, over time, produce calcification. We cannot model this full and complex system in vitro because at the moment it is not possible to recreate the tissue and the interaction of this whole tissue and platelet delta granules in vitro.

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 objective is to ascertain whether there exists a statistically significant distinction between groups. The variable under consideration is the presence of calcification. If we detect calcification, we will label the mouse as having calcification. Conversely, if no calcification is detected, we will classify the mouse as lacking calcification.

The presence of calcification in a mouse is a binary response, categorised as either "yes" or "no". We will tally the number of responses and employ Fisher's exact test to determine if the disparity in calcification probabilities between the two treatment groups is significant.

Therefore, since this is a binary response, we will need 4 animals for each group (see the Scientific Background session for more details on how the number of animals was calculated).-

We will need animals on a high-caloric diet (known to stimulate cardiovascular calcification) and on a regular diet.

Finally, since calcification is known to increase with age, we will need animals at two time points: at 12 months, and at 18 months old.

With all these variables and considering that we need a minimum 4 animals per group to have statistical confidence in the differences between them, and the fact that the animals will be bred we will need a total of 130 animals for the whole project.

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

We considered firstly what would be the essential information for this project to be successful. Based on this, we calculated the minimum number of animals necessary to confirm or refute our hypotheses. In our project, the response to the intervention studied is a binary response, and therefore we used as statistical analysis tool Fisher's exact test. This provided us with a minimum number of animals to determine whether delta granules are indeed responsible for the presence of calcified particles in cardiovascular tissue. Besides the minimum number of animals for this project to be successful we took into account the number of animals necessary for the breeding program.

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

As the number of animals used in this project is relatively small, a pilot study would unnecessarily increase the number of animals to be used on this project rather than optimise it.

We will also look for animals of the same genetic background being used on other projects and, if found, we will ask for the tissue to be shared with our project. We will be using wild type animals and APOE-/-, which are regularly used in research under similar diets, if not identical to the one necessary for this 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.

The mouse chosen for this project is the only model that reproduces a human condition where platelets do not have delta granules. This mouse model perfectly reproduces the lack of delta granules as seen in humans and is the perfect model to determine whether delta-granules are responsible for cardiovascular calcification or conversely, if the lack of delta-granules is responsible for the lack of cardiovascular calcification.

The animals will not be subject to any methods other than one blood collection (for example 100 microliters of blood, by tail vein collection, as this method is considered appropriate based on the volume collected and the safety for the animals.) in a lifetime to evaluate the degree of absence of delta granules. One group of animals will also be subject to a high fat diet until the end of this experiment.

Certain animals used in our study will be genetically modified, and they may exhibit a higher risk of blood coagulation problems, with coagulation times approximately 7 times longer compared to wild- type animals. In addition to their increased susceptibility to bleeding due to the mutation, these animals may also have an elevated likelihood of developing fibrotic restrictive lung disease (decreasing the capacity of the lungs) and potentially developing tumours as they age with no higher incidence than what is observed on the wild type animals (around 1% of animals presenting tumours at the age of 18 months).

To ensure their well-being and minimize potential risks, we will closely monitor the animals for any signs of injury that may occur within their enclosures. We will constantly assess any variations in their physical condition within different compartments. We will regularly monitor the animal's weight to identify any unusual fluctuations. Moreover, due to the high risk of prolonged coagulation times, we will discuss with the NACWO and NVS the possibility of keeping the males individually housed to prevent fighting.

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

The mouse chosen for this project is the only model that reproduces a human condition where platelets do not have delta granules. We will need animals on a high caloric diet (known to stimulate cardiovascular calcification) and on a regular diet.

Finally, since calcification is known to increase with age, we will need animals at two time points: at 12 months, and at 18 months old.

No procedure will be done on the animals besides one blood collection (5% of total animal blood) in a lifetime and a high fat diet, which a subset of the animals will be subject to until the end of the experiment.

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

The animals will be monitored regularly (aiming for daily and no less than 4 times a week) using a scoring system. Individuals will be trained to identify signs of distress and health issues, and their competence will be assessed in collaboration with the NACWO and NVS.

Any welfare issue will be immediately brought to the attention of the NACWO and NVS and

mitigations will be applied.

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

We are following the NC3R’s best practice guidance and ARRIVE and PREPARE guidelines.

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

I am in constant contact with the NVS and NACWO to keep updated on the best animal work practices. Also, and with the help of the NIO, I will be actively looking for new courses related to advances and best practices in the NC 3R's.

9. What Drives Melanoma Spread and Testing New Treatments for Melanoma and Ovarian 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, immunity, immunotherapy, metastasis, melanoma

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 understand how gene expression and metabolic changes enable melanoma to adapt to new tissue environments and develop drug resistance, with the goal of developing and validating new therapeutic options. Where relevant, these treatments will also be evaluated in preclinical models of ovarian 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?

Each year in the UK, around 17,000 people are diagnosed with melanoma, leading to over 2,000 deaths annually. Additionally, over 7,000 new cases of ovarian cancer are identified, with more than 4,000 deaths each year. For most patients with advanced-stage disease—when the cancer has spread to other parts of the body—there is often little hope of a cure,

and survival may be limited to a few years or even just months after diagnosis. Despite these challenges, advances in understanding the molecular, cellular, and metabolic processes that go wrong when cancer spreads have led to the development of drugs and cellular therapies that can significantly slow disease progression and, in some cases, even achieve remission. However, the number of these effective treatments remains limited, and treatment resistance continues to be a major obstacle. Expanding the treatment options and finding ways to overcome resistance are critical areas where further research is urgently needed.

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

By the end of the project, we aim to have validated several new potential therapeutic targets for treating advanced melanoma or ovarian cancer, which may also be relevant to other cancers.
Additionally, we seek to understand how cancer cells develop resistance to immune checkpoint inhibitors—drugs that enhance T cell responses (T cells being a key part of the immune system). We will explore whether these resistance mechanisms can be reversed using other drugs. Further, we plan to design and evaluate the safety and effectiveness of a novel chimeric antigen receptor (CAR). CARs are built from multiple proteins and introduced into a patient's T cells, guiding these cells to recognize a specific protein on the cancer's surface and then activating the T cells to destroy the cancer cells. Our findings will be shared at scientific conferences and in research publications, and any newly developed reagents will be licensed for commercial development where appropriate."

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

Our research is driven by a commitment to improving outcomes for cancer patients. Over the next five years, we expect that scientists in academia and the pharmaceutical industry will build on the knowledge we generate and share, using it to gain deeper insights into tumour immune responses, including the development of immunological memory, and to create new treatments and diagnostic tools. However, we recognize that any new therapies arising from our discoveries may take several years to reach patients, as they must first be identified and undergo thorough testing before they can be safely prescribed.

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

We will maximize the impact of our work through active collaboration and by sharing our findings as early as possible, first via pre-print servers and then through Open Access journals. Aligning with our institution’s policy, we commit to making negative findings publicly available when appropriate and feasible. Specifically, we will aim to publish negative results in cases where the findings hold scientific value, particularly when they may inform and guide the research community, reducing the likelihood of unnecessary repetition of animal-based studies. This commitment recognizes the importance of rigorous experimental controls and considers both practical challenges and the availability of publication avenues for such data. Additionally, we will present our research at international conferences attended by academics, clinicians, and industry professionals. Our current partnership with a biotech company allows us to directly transfer our knowledge to an organization equipped to develop cellular therapeutics and advance them into clinical testing.

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 adult mice for our studies because the mouse immune system has been extensively researched, making our understanding of it the most advanced among laboratory animals. The necessary reagents for working with mice are readily available and well-tested, unlike those for many other species. Mice accurately reflect the human immune system in immunotherapeutic studies, and correlative studies show that results can often be translated between the two species. Adult mice have a mature immune system, are large enough to host tumours, and are more practical for treatment, allowing them to tolerate side effects better than younger mice. Additionally, we will use mouse embryos to live-image the migration and invasion of fluorescent-reporter-tagged melanocytes and melanocyte precursors—cells that produce skin pigment responsible for tanning and hair colour. We hypothesize that important similarities exist between these cells and melanoma cells as they disseminate through solid tissues.

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

Some animals will be used solely for breeding purposes. In most animals, we will establish tumours, primarily melanoma, at both superficial and internal sites. We will track tumour development over several weeks to months, often using non-invasive whole-animal imaging techniques. Certain animals will receive therapies, including drugs and cellular treatments, by injection, by a feeding tube reaching the stomach or in their food or water, and we will closely monitor their responses over days to weeks to assess both the effectiveness of the therapy and any potential side effects. Monitoring may require non-invasive imaging that would require anaesthetising the animals. Strong evidence suggests that melanoma cells become more like embryonic cells during disease progression or in response to treatments. Although we do not yet fully understand the functional significance of this reversion, it is likely to influence how tumour cells interact with the tissue environments they invade and with immune cells. To gain deeper insights into this process, we will conduct live imaging studies of embryonic development.

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

Cancer growth within an animal, as in a human, can have negative effects such as weight loss and obstruction. However, we closely monitor weight and specific behaviours in the animals to ensure their well-being, implementing observation schedules, control measures including appropriate anaesthesia and analgesia, and humane endpoints to minimise any potential discomfort. Genetically altered mice developing tumours or serving as hosts for transplanted tumours exhibit a sub-threshold or only mild phenotype when bred to maintain a colony. Tissue sampling from the ear (a non-regulated procedure) may be necessary to evaluate the genotype of mice and will be undertaken by highly trained staff. The planned treatments will use the least invasive route possible to administer the agent at doses which avoid toxicity; more invasive administration such as by feeding tube will be undertaken by highly trained staff. In some cases, we may expose only a few mice to an

unknown agent, escalating the dose with careful monitoring. If our monitoring indicates any adverse effects, we will promptly adjust the therapy dose or discontinue treatment, and we will humanely kill animals to avoid lasting harm. For imaging embryos, we will use mice wherein genetic alteration labels their melanocytes with a fluorescent protein. No adverse effects are expected from substances administered to pregnant mice to switch on the fluorescent protein, nor from the expression of the fluorescent protein itself. Prolonged imaging of embryos will require extended anaesthesia. Sufficient and terminal anaesthesia should mitigate any adverse effects experienced by the mice; additionally, signs of vitality (regular heartbeat and body temperature) and hydration will be monitored regularly and supported to ensure no mice undergo unnecessary stress. If signs of stress are observed, mice will be humanely killed to avoid lasting harm. At the end of our experiments, animals will be humanely killed, and tissues may be collected for further laboratory 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)?

Minimal, if any, adverse effects are anticipated in the animals used purely for breeding. All procedures involving animals with tumours, or without tumours will be managed to ensure they do not exceed moderate severity, and most will experience mild severity as we will limit the growth of tumours to avoid interfering with vital functions or causing pain and use safe doses of agents. Imaging of embryos is classified as a non-recovery procedure. Overall, we estimate that the lifetime experiences of mice on this licence will be distributed as follows: approximately 35% will experience sub-threshold severity, 50% will experience mild severity, and 15% will experience 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?

The multiple cell types implicated in cancer formation, progression, and treatment response are all present only in an animal host. Additionally, in the case of drugs or cell therapies, we intend to address whether the therapeutic will be absorbed, metabolised and distributed effectively to have their effect without causing significant toxicity. Ultimately this requires an intact, living organism (notwithstanding the caveat that mice and humans have important pharmacological differences).

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

Human tumours and tissue culture were considered as non-animal alternatives and will be used extensively before animal experiments.

Why were they not suitable?

Non-animal alternatives cannot be used to test all our hypotheses due either to our inability to manipulate the system (as with human tumours, outside the context of a clinical trial with its own attendant ethical issues) or as with tissue culture because we cannot yet replicate all the important features of the in vivo environment and certainly not holistically. Co-culture systems can begin to address interactions between different cell types a couple at a time but not the entire spectrum of interactions. In particular, immune responses require the orchestrated activities of multiple cell types located at distinct anatomical sites, with various cells needing to migrate from one anatomical site to another.

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 each experiment, pilot and historical data are used in power analysis to predict optimal group sizes. Numbers are then scaled to encompass the anticipated/desired number of experimental scenarios, incorporating controls, different doses and independent replication.

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

Experiments will be appropriately controlled and mice of the same age, genetic background and source used to reduce the variability and thereby produce highly consistent data. An on-site statistician has advised us on design and power analysis, alongside the use of the NC3Rs Experimental Design Assistant. Bias will be avoided by random allocation of animals to treatment groups. Where possible and practicable, endpoints will be assessed by a technician who is unaware of the treatment the animal has received. Experiments will be designed carefully to avoid other sources of bias such as week-to-week or operator-to-operator.

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 is used to minimise the sizes of colonies of genetically altered mice and to generate the numbers required for experiments. Real-time monitoring of cells (e.g. luciferase mediated imaging of tumour cell development) has considerably reduced the group sizes required for significant experimental results.

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 provide an in vivo context (anatomy, physiology, metabolism) that is relevant to human cancer and can be manipulated to generate data applicable to human cancer treatment. Therefore, mice are the most suitable in vivo model for achieving the stated objectives.

Sampling blood and administering tumour cells or drugs via subcutaneous, intraperitoneal, or tail vein injections are procedures performed by highly trained staff, ensuring brevity and minimizing discomfort. Needles will only be used once for sampling or administering substances to the mice. By responsibly considering the potential adverse effects associated with these regulated procedures, mechanisms are in place to minimize discomfort, including appropriate anaesthesia and analgesic regimens for pain avoidance and relief.

To further reduce suffering in tumour-bearing animals and treated animals, they will be humanely killed as soon as tumour formation is sufficient to yield satisfactory data, and always before they exhibit severe pain, or lose significant weight, which will be closely monitored.

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

Cancers develop in vertebrates, and mice are more similar to humans than lower vertebrates, such as zebrafish. This similarity is critical for using reagents, like drugs developed for human targets, and for translating findings back to the clinic. Since cancers develop over many weeks to months, the use of terminally anaesthetized animals or immature mice is not practical. Additionally, immature mice lack a functional immune system, which is essential for our research.

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

Therapeutic agents may be associated with toxicity related to the underlying mechanisms of anti- tumour activity and off-target effects. Our experience indicates that this has most frequently manifested as weight loss in less than 20% of experimental animals. This issue has been effectively managed through close observation and by providing mashed food instead of hard pellets. Whenever possible, animals will receive analgesia as outlined in the relevant protocol to control adverse symptoms associated with the treatment. In all cases, a predetermined set of criteria will be used to assess whether the duration of these symptoms is excessive or if the animals appear stable and/or show improvement over time, suggesting that these effects are transient.

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

PREPARE guidelines will be consulted ahead of initiating animal experiments.

All tumour studies will be conducted in accordance with the guidelines for working in

rodent models of cancer as described by Workman et al (2010). (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. Br J Cancer. 2010 May 25;102(11):1555-77).

LASA guidelines will be followed regarding volume of blood samples, and drug dose volume limits.

ARRIVE guidelines will be adopted when reporting our findings.

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 my NACWO, NVS, colleagues and HO inspectors.

10. Molecular Regulation of Mammalian Development

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

oocyte, fertilisation, embryonic development, histone, epigenetic reprogramming

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 objective of this project is to increase our understanding of the molecular processes regulating mammalian embryo development. Particularly, we want to study the function of specific genes involved in this process.

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 discovery that certain factors can ‘reprogramme (transform)’ mature cell types into stem cells holds great potential for new biomedical applications, such as cell replacement, drug testing and disease research. Reprogramming allows us to turn any cell of the body into a stem cell. However, the mechanisms involved in this technique are only just being identified and the success rate of the method remains very low. One way to improve our understanding of reprogramming is to study the natural programming mechanisms that begin after fertilisation in the mammalian embryo. In addition, basic scientific discoveries resulting from this programme of work will provide knowledge of the molecular processes during fertilisation and early embryonic development. It would benefit couples undergoing reproductive technologies such as in vitro fertilisation in the future.

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

We aim to study the onset of the gene expression event in mouse embryos. We aim to uncover new factors that control early embryonic development and further investigate their roles in the embryos. Early embryo has the potential to “reprogramme” their genome and restart the developmental processed to become all the cell types in the body (totipotency). This work will bring novel information about gene function at the beginning of development.

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

Identification of molecular mechanisms will lead to a greater understanding of how totipotency is achieved/maintained and has important implications for reproductive technologies and areas such as regenerative medicine.

The long-term potential benefits of this study are that data generated may have far-reaching implications for the regeneration of cells and tissue through stem cell reprogramming and differentiation, allowing repairing the damage tissue/organ.

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

Findings will be made available to other scientists through publication in peer-reviewed, open access journals and presentations at scientific conferences and meetings nationally and internationally. The animals generated will be provided new insights on early embryonic development and we will make available to other scientists.

Species and numbers of animals expected to be used

- Mice: 6,050

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: The mouse is selected as a model species as there is more information available about this species than any other in genetics, molecular biology and reproduction.

To investigate fertility issues, we need adult mice to produce the oocytes/embryos we need for our experiments.

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

Our project includes hormone Intraperitoneal injections. The purpose is to use hormone to stimulate the ovary to generate more oocytes within a cycle.

For in matured oocyte collection, only one hormone (Pregnant Mare’s Serum Gonadotrophin, PMSG) injection needed. To induce super ovulation, a second hormone injection (human Chorionic Gonadotrophin, hCG) will apply 48 hours post PMSG inaction.

We will perform surgical procedure in the “Embryo Recipient” procedure.

Duration: 15-30 mins per recipients.

Procedures:

1. Recipients for embryo transfer will be rendered pseudo-pregnant by mating with a vasectomised sterile male
2. Under general anaesthesia a single/bilateral flank or midline laparotomy will be performed, and genetically modified embryos will be implanted into the oviduct or uterus (AB).

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

In order to provide eggs and early embryos, female mice will be given hormones to maximise the number of eggs and embryos produced. They are then killed by a humane method and the embryos collected. During the procedure these animals will only experience transient pain at the time of injection. Using molecular laboratory techniques, we will identify and characterise the key factors involved in the regulation of gene expression during early embryo development in vitro – so the vast majority of the mice we will use are bred and killed for egg and early (up to day 4) embryo harvest and not subject to any invasive procedures.

The ability to alter specific genes in the laboratory provides researchers with the opportunity to study the function of a particular gene. We will culture the eggs and embryos in specialist culture systems; assess their development and the effect of modified gene function. Very occasionally we may need to perform surgery on the mice, under general anaesthesia (for example to transplant some embryos into female mice or to perform a vasectomy on male mice). These are essential techniques (with moderate severity) but good surgical techniques, anaesthetic and pain relief will be used during and after surgery to minimise adverse effects. The mice are allowed to recover and will monitor closely post-operatively. Some mice will deliver at term and other pregnant females will humanely be killed at specific time points in early gestation. At the end of the study all the mice will be humanly killed by an approved method.

We will also breed genetically altered (reporter) mice (to obtain eggs and early embryos). These mice have been generated under other project licences. The effects of the genetic alterations (a fluorescent marker tagged to a protein of interest) are negligible, and the animals suffer no adverse side effects of this alteration. These mice will be superovulated and mated to obtain early embryos, experiencing no more than the same minor discomfort at the time of injection, as the control mice described above.

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 breeding and maintenance of genetically modified animals (Protocol 1) should not generate any severity level.

All mice undergoing superovulation (Protocol 2) will experience mild severity levels.

All mice used as embryo recipients will (Protocol 3) will experience moderate severity levels.

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?

We will use mice mainly as oocyte/embryo donors with the remainder of the experimental work being carried out in the laboratory in vitro. There is no alternative to the use of animals for the basis of our work.

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

We will firstly use in silico analysis by analysing published bioinformatic data to shortlist the number of genes of interests to minimise the usage of animals used. We will also use cell culture to perform biochemical analysis to replace animal experiments.

Currently, the technology of generating mouse oocytes and embryos from stem cells is still in the experimental phase and many procedures need to be optimised. We will use this alternative when the technology is matured.

Why were they not suitable?

We cannot obtain good quality oocytes or embryos without the use of 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?

Wherever possible, attempts will be made to reduce the number of animals used in order to address specific scientific questions, while making maximal progress in achieving the goals of the project. Experimental design is given priority, with regular consultation between all members of our collaborative groups. Numbers of animals used in each experiment are based on estimates of variability expected from our previous experience or from published data. Appropriate positive and negative control treatments are included where necessary. All procedures are carried out by highly- trained staff to maximise the success of vulnerable procedures such as oocyte/embryo microinjection and nuclear

transfer.

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

Firstly, we will use superovulation methos to increase the yields of oocytes and single cell embryos (zygotes) to reduce the usage of mouse donors. The numbers of samples recovered by superovulation treatment is approximately fivefold.

We will apply Experimental Design Assistant (EDA) tool to perform power calculations to optimise the number of animals will be required to reduce the numbers of animal usage. For example, our previous experience using this technique in our previous PPL has shown that at least 30 embryos should be successfully injected for each group. This number is obtained from a power analysis calculating that a sample size of 30 embryos per group will permit observation of a 5% significant difference in measured values, based on prior standard deviation.

We will also apply suitable statistic methods fit for various experiments, for example, differences in gene expression will be assessed by quantitative PCR (data analysed by Student's t-test and ANOVA). Protein levels will be measured by Western Blotting and/or immunofluorescence, or biochemical methods – such as chromatin/methylation analyses.

We will also use molecular techniques that allow assay of single oocytes and embryos (and in some instances single blastomeres) reducing the degree of replication and numbers of observations required.

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

Careful statistical treatment of all data will be undertaken, gaining as much information as possible from each experiment. Whenever feasible, experiments will be consecutive to allow dissemination of results from previous experiments to influence current experimental design. Power analyses will be conducted prior to new experiments to determine an appropriate sample size to achieve adequate statistical significance. Proper trainings will be introduced to reduce experimental losses when manipulating embryos (e.g. for embryo micromanipulation).

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 selected as a model species for these studies for several reasons. Firstly, there is more information available about this species than any other in genetics, molecular biology and reproduction. Secondly, the short reproduction interval allows

studies to be completed more quickly than in any other mammal. Finally, more consistent observations can be expected as inbred strains are maintained in a closely controlled environment. All mice undergoing surgery will receive pain relief (analgesia) and good post-operative care. Moreover, all of our animals are housed under pathogen free, environmentally controlled conditions. Animals are routinely monitored for the presence of pathogens that could potentially lead to infections.

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

We have been using the most suitable, standard reproductive ages (from 5 weeks) for our experiments. Animals at an immature life stage will not have suitable experimental materials (i.e. oocytes/embryos).

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

We have relocated our animals to a brand-new IVC facilities with additional measures (e.g. air shower, pressure control etc.). Based on the breeding and less-distress performances, it shows that we have improve their welfare.

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

We have adapted the most refined method to induce ovulation by using a new "ultra superovulation" method by injection of inhibin antiserum with gonadotropin. This method will boost the oocyte/embryo harvest by ~2-3 folds which can significantly reduce the animal usage.

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

We will check related references, attend 3Rs meetings.

11. Molecular basis of Mammalian Development 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

embryonic development, organs, neurodevelopment, neurodegeneration, stem cells

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?

Our aim is to understand the mechanisms underlying embryonic development as well the maintenance of stem cells and organ formation in adult organisms. We also aim to understand how diseases develop when these processes go wrong.

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 findings from our research will provide information on how organ formation takes place during embryonic development and how stem cells are maintained in organs such as the brain and the intestine. This information will have applications in developing better methods of converting adult cells into stem cells, in tissue engineering and repair. In addition, deregulated developmental processes can give rise to diseases. Mouse models

for developmental and disease processes will help unravel disease mechanisms and have applications in evaluating therapeutic approaches.

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

Research conducted under this PPL will

- provide new information on the mechanistic understanding of neuro-developmental disorders and late onset neurodegenerative diseases which will be communicated for publication in high impact peer reviewed journals.
- be presented at international, national conferences and workshops

Expected benefits from studies on 'how knockout of/or mutations/deletions in proteins that form the basal body of the primary cilia cause neuro-developmental disorders (ciliopathies) and motor neuron disease.'

The cells in our body have a component called primary cilia which consist of an antenna like projection sticking out of the cells and a structure called the basal body that anchors it to the cell. In diseases called ciliopathies, cilia are abnormal or absent. Primary cilia are also essential for the formation and survival of spinal nerves. In motor neuron disease spinal nerves die eventually leading to death by asphyxiation. The basal body consist if a large number of proteins which form a complex. Talpid3, Nek1 and c21orf2 are proteins which form the basal body.

What we hope to find from our research are:

1. The molecular and cellular changes associated with alterations in basal body proteins such as Talpid3 seen in Joubert syndrome (JS) which is a ciliopathy (neurodevelopmental disorders).
2. How genetic alterations in basal body proteins such as Nek1 and C21orf2 cause motor neuron disease (a neurodegenerative disorder) in which nerves from the spinal cord die.
3. The molecular mechanisms that links stem cells and neurodevelopmental/neurodegenerative disorders and find out if these diseases share some common mechanisms.

Expected benefits from studies on 'how DNA binding proteins such as Caudal, Polycomb group and Trithorax group regulate stem cells in the brain and intestine.'

DNA binding proteins are found in the nucleus of the cell and they are important for regulating the expression of genes. Some of these genes are critical for developmental processes including the vertebral column , nervous system and the intestine.

What we hope to find from our research:

1. Studies such as the ones proposed for *Caudal* (*Cdx), Polycomb Group (PcG)*and Trithorax Group (*Trx-G)* genes will provide new insights into the molecular basis of the regulation of stem cell identity and function in adult tissues such as the intestine and brain. The outcomes will be a greater understanding of how tissues in the body can replenish with implications for tissue repair and tissue engineering.
2. Data from such *in vivo* experiments will enable us to design and perform *in vitro* experiments to dissect the molecular mechanisms of the function of these genes.

3. Experiments such as those on the skeletal patterning mutants will provide an understanding of the molecular basis of human birth defects which are developmental lesions.

Expected benefits from 'What is the molecular basis underlying the pleiotropic role of Angiogenin in neural development, neurodegeneration and stem cells'.

Angiogenin is a protein that is important for the growth of nerves and blood vessels and also for stem cells in the brain.

1. Studies proposed under this objective will provide new insights into the molecular basis how stem cell are maintained and function in adult tissues such as the intestine and brain. The outcomes will aid in understanding tissue regeneration with implications for tissue repair and tissue engineering.
2. Data from such *in vivo* experiments will enable us to design and perform *in vitro* experiments to dissect the molecular mechanisms of the function of this gene and the mutations found in motor neuron disease patients.

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

The proposed research will lead to a better understanding of (1) stem cell regulation; (2) ciliopathies, a group of neurodevelopmental disorders classified as rare diseases which are ill understood and; (3) late onset neurodegenerative diseases such a motor neuron disease.

Our research findings and mouse models will

- be of interest to the rare diseases patient interest group
- be of interest to patients with motor neuron disease
- have applications in therapeutic evaluation.
- have implications for regenerative medicine and generation of cells with specialised functions such as nerves with applications in tissue repair, regeneration and wound healing.
- will help develop better approaches to the treatment of neuro-developmental and neurodegenerative diseases
- The genetically modified animals will provide disease models which will be of interest to pharmaceutical companies for evaluation of therapeutics.
- Our research will also be of interest to clinicians and pharma.

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

- Based on our findings we will seek to collaborate with tissue engineers and clinicians for appropriate applications of our findings on stem cells, motor neuron disease and ciliopathies.
- We will present our findings in scientific conferences and also highlight any unsuccessful approaches.
- Successful application of the 3Rs will be disseminated through NC3Rs.
- We will publish our findings in peer reviewed journals.

Species and numbers of animals expected to be used

- Mice: Estimated number of mice annually is 850 which will include the homozygote,

wild type and heterozygote mice generated through breeding protocols.

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.

Models: The mouse has been chosen as the model in this study as it is the lowest vertebrate group in which studies of organ development, stem cells and patterning can be carried out. M.ice share many similarities with humans since they are both mammals. The developing mouse embryo is an extensively used model system for determining the role of normal and abnormal genes in developmental processes.

The mouse embryo is a good model system for understanding vertebrate development for the following reasons:

a) well characterised genetic markers are available and the information from genome sequencing allows identification of functionally active genes.
b) the gestation time of the mouse is reasonably short.
c) conditions have been well worked out for the isolation and culture of pre-implantation and early post- implantation mouse embryos.
d) it is possible to introduce new or modified genetic information into mouse embryos that could perturb development or serve as markers to trace the cell lineages and follow expression of a particular gene product.
e) it is possible to introduce the normal or genetically modified embryonic stem cell (ES) or cells from the inner cell mass into mouse blastocysts in order to derive transgenic mice that carry an altered gene.

The mouse models we are using to study the role of the basal body/ cilia proteins are conditional knockouts thereby minimising the number organs or stages that are affected.

Stages: Since we are investigating cellular and molecular mechanisms of neurodevelopmental disorders, we will use animals at the stages showing the earliest signs of clinical manifestation thereby minimising suffering and distress.

For studying genes that regulate development, stem cell identity and maintenance, in self renewing tissues as intestine and in organs such as the brain, we will use embryonic, peri and postnatal stages as well as adult mice from strains which have been genetically modified. Such studies will provide an understanding of how these genes regulate developmental processes.

For studying genes that have been shown to be mutated in motor neuron disease by Genome wide analysis studies (GWAS) we will use post-natal, juvenile and adult mice which have been genetically modified. This will enable to study early molecular and cellular changes before the overt manifestation of the disease.

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

Most of the strains we propose to use have already been generated by us or others. In addition, we will be generating a few lines with human disease specific changes. For this

we will inject hormones into female mice to induce superovulation, obtain preimplantation embryos or fertlized eggs and inject these eggs/embryos with genetic material, transfer them to foster mice and allow the embryos to come to term. These offsprings will be checked to see if they carry the altered gene and bred for experimental purposes as described below.

Pre-implantation stage embryos obtained by superovulation of females and normal mating or sperm from males will be used for cryopreservation.

Embryonic stem cells will be derived from pre-implantation embryos isolated after superovulation.

More typically we will mate genetically altered mice and obtain embryos or tissues after humane killing of the mice. We may, for some strains, induce gene expression by either administering Tamoxifen or tetracycline and then isolate tissues or embryos after Schedule 1 killing.

The cells, tissues or embryos will be analysed for cellular, molecular and biochemical changes using histology, immunohistochemistry, and high throughput analysis of gene and protein expression.

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

Some of the DNA binding protein knockout mice are smaller than their normal litter mates, have defective vertebrae and stem cell defects in the brain and gut. They groom and feed normally and we typically kill these by Schedule 1 method at the first signs of distress, weight loss or pain.

The genetically altered basal body protein mutant mice show signs of ataxia at around post natal day 15, such as righting themselves slowly in comparison to their wild type litter mates and starting to drag their hind limbs. They also start to show a domed head at around day 15. However, they are able to groom themselves and are able to suckle. These mice are killed before the ataxia progresses i.e. at post-natal day 15 or earlier, since beyond 15 days the ataxia progresses and animals are in distress by day 20.

For the new strains that we propose to develop for patient specific motor neuron disease/ciliopathy risk factors and for their knockouts we cannot predict the severity of the phenotype until they are generated. Once generated these animals will be monitored regularly for adverse effects and killed by Schedule 1 method if they show signs of distress.

One of the knockout mouse strains we propose to obtain from a collaborator shows no overt pathological phenotypic changes. These mice breed and feed normally but mice over 9 months of age develop defects in white blood cells called leukopenia. In this case the mice will be killed by Schedule 1 method before the onset of this phenotype.

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)?

Under breeding and maintance of GAA, all the reporter transgenic strains such those expressing epitope tags, fluorescence tags, APEX , the Cre driver strains and conditional knockout mice with LoxP sites that we will use, have no adverse phenotypes and so fall under the mild severity band.This is because they are not expressing any harmful mutations.

Currently 25% of mice from five of the current genetically modified mouse lines that we use show moderate severity as either they have gut or brain defects .

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?

Organs are not uniform populations of identical cells but are composed of different specialised cell types and have a 3-dimensional organization which depends on complex cell interactions. Hence, any analysis of the coordinated regulation of cell multiplication and specialisation as well as organisation in any organ needs to be carried out in developing embryos and adult organisms. This is because such processes occur within a defined anatomical three dimensional tissue architecture containing the multiple cell types. Similarly, in order to study stem cells within an organ and how these are maintained, an animal model is most appropriate.

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

Wherever possible, for evaluation of gene constructs and preliminary studies, we already make use of tissue culture models. We use embryonic stem cells to evaluate our gene editing approaches by CRISPR/Cas9 prior to applying them to mouse embryos.

We have also been developing 3-dimensional organoid models for carrying out biochemical studies using human induced pluripotent stem (iPS) cells or mutant mouse ES cells . I had been funded by a project grant (BRACE and NC3Rs) for developing patient specific induced pluripotent stem cells as a disease model to reduce and replace animal models of motor neuron disease.

We also use early zebrafish embryos (stages upto 120hpf which are not regulated) for gene construct evaluation.

Why were they not suitable?

Tissue cultures although excellent to carry out biochemical and molecular biological characterisations of the functions of genes and genetic pathways are not entirely appropriate for our studies. In undertaking studies such as on mammalian development or

to study late onset neurodegenerative diseases which cannot be fully recapitulated in cell cultures we have had to consider the use of laboratory mice .
We have developed brain organoids from human iPS cells as a model for Fronto temporal dementia (Ferguson et al 2024). However, studies related to motor neuron disease or Alzheimer's disease, the cell culture/ organoid models do not have the necessary complexity to model the disease fully. In the future we would like to perform studies on behaviour and locomotion in the context of these diseases for which cell culture models are not suitable.

In the context of stem cell homeostasis of the intestine, although intestinal organoids are in use, the mammalian intestine matures postnatally and for the purpose of the questions we wish to address, organoids can only partly substitute 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 gene knockout mice will be maintained as heterozygous lines. To generate homozygous experimental animals heterozygous crosses will be set up which will produce offspring in Mendelian ratios ie 25% will be homozygotes, 50% will be heterozygotes and 25% wild type.
- The Transgenic flourescent reporter, cell cycle reporter and Cre deleter mouse strains will be maintained as hemizygous lines.
- The conditional knockout mice ie floxed mice will be maintained as homozygous lines.

How we have arrived at the proposed number of 6 mice of each genotype for phenotypic analysis .

For the new GAAs that we will generate and for the crosses with reporter or Cre strains of mice, we cannot fully estimate the phenotypes and standard deviations of the intercrosses. Hence we have used a crude method of calculation based on the law of diminishing return.

This method is called “resource equation” method. This method is used when it is not possible to assume about effect size, to get an idea about standard deviation as no previous findings are available or when multiple endpoints are measured or complex statistical procedure is used for analysis.

According to this method a value “E” is measured, which is nothing but the degree of freedom of analysis of variance (ANOVA). The value of E should lie between 10 and 20. If E is less than 10 then adding more animals will increase the chance of getting more significant result, but if it is more than 20 then adding more animals will not increase the chance of getting significant results. Though, this method is based on ANOVA, it is applicable to all animal experiments. Any sample size, which keeps E between 10 and 20 should be considered as an adequate. E can be measured by following formula:

E = Total number of animals − Total number of groups

In our case E=12 (6+6) and total number of groups is 2 with E=10 and therefore the numbers we have proposed is adequate. Once we have the mice and analysed the phenotype we will be able to do power calculations to confirm that these numbers are sufficient.

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

- Since we are using GAA for our experiments it is not easy in all cases to predict the numbers we will generate simply because we will not only generate homozygotes and wild types to be used in our analysis but also heterozygotes many of which in the case of our mouse strains show no phenotype.
- For each strain, the breeding nucleus will be small consisting of 2-3 breeding pairs replaced every 4 months.
- For the functional and histological assays we propose to compare at least 6 homozygotes from postnatal, adult and aged mice with their wild type (WT) type littermates if the mutation is fully penetrant as is the case with most of our strains.

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 the use of mice and mouse embryos for preliminary studies we use cell cultures and embryos of lower vertebrates such as zebrafish (stages up to 5d post fertilization which are non- regulated). The findings will then be confirmed using the mouse so that we only have to use the most appropriate and minimal number of constructs on a small number of mouse embryos and sacrifice animals at only the required stages.

Our breeding nucleus is generally small between 2-3 breeding pairs and colony numbers are managed efficiently to keep mouse numbers low.

We will also use refined gene editing technologies such as the CRISPR/Cas9 system to generate knockdowns/knockouts and transgenic mice as these are emerging as more efficient technologies which will reduce the use of animals.

At appropriate places in the protocols, I have indicated the *in vitro* experiments that will be performed.

All of the mouse experimental approaches used are standard and well established techniques in my laboratory so failure of experiments is not very common.

We can access the services of statistician who we will consult when required , although the current developmental biology experiments we propose do not involve complex statistical evaluations.

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 mouse has been chosen as the model in this study as it is the lowest vertebrate group in which studies of organogenesis, stem cells and patterning can be carried out as they share many similarities with humans. The developing mouse embryo is an extensively used model system for determining the role of normal and altered genes in developmental processes.

The mouse embryo is a good model system for understanding vertebrate development for the following reasons:

a) well characterised genetic markers are available and the information from genome sequencing allows identification of functionally active genes
b) the gestation time of the mouse is reasonably short
c) conditions have been well worked out for the isolation and culture of pre-implantation and early post- implantation mouse embryos
d) it is possible to introduce new or modified genetic information into the pre-implantation and early post-implantation embryos that could perturb development or serve as markers to trace the cell lineages and follow expression of a particular gene product
e) it is possible to introduce the normal or genetically modified embryonic stem cell (ES) or cells from the inner cell mass into mouse blastocysts in order to generate injection chimeras and eventually derive transgenic mice that carry an altered gene.

The mouse models we are using to study the role of the basal body/ cilia proteins are conditional knockouts which are either temporal or spatial knockouts thereby minimising the number organs or stages that are affected.

Since we are investigating cellular and molecular mechanisms we use animals at the pre-clinical and at the stages showing the earliest signs of clinical manifestation thereby minimising suffering and distress.

Methods

The majority of our methods for analysing the mutant mice are of mild severity which either involve isolating fetal forms or tissues from post-natal, juvenile or adult mice by Schedule 1 killing to anlayse the cellular, molecular and biochemical changes . These cause minimal suffering and distress.

Our methods for investigating cell cycle/proliferation and protein synthesis are minimally invasive.

Conditional gene expression is also a refined approach to understand gene function since we turn off/on the gene only in specific cell types or at specific times in development or life cycle.

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

We already use zebrafish embryos (up to d5 post-fertilization) or cell cultures to evaluate constructs and cellular phenotype prior to generating transgenic mice. Organs such as the

hind brain (cerebellum) develop post-natally and the intestinal epithelium matures post-natally and in such cases mice are appropriate models .

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

Mice will undergo minor surgery for vasectomistaion or during embryo implantation under anaesthesia and are expected to recover quickly. They will be adminitered painkillers and post operative care just like people recovering in a hospital. They will be monitored regularly to ensure that they are not suffering from any infection or problems with wound closures.

We will use cell type and tissue specific knockouts i.e. conditional KOs to minimise the phenotypic consequences of gene deletions or over-expression.

Stock lines which show no adverse phenotype as for example - Reporter or Cre expressing mouse strains are maintained as homozygous line. Other strains which show adverse phenotype in the homozygous state are maintained as heterozygous lines. These contribute to maintaining smaller number of mice in the colony.

New mouse mutant lines that we will be generating will have very well defined monitoring scheme to identify stages and extent of suffering and distress.

In the case of newer transgenics or KOs mouse lines yet to be generated, we will consult with the NAWCO, NVS and Home Office inspector to decide on the steps for minimising distress and a humane end point that will allow functional analysis without undue distress to the animal.

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

We will use the Experimental design assistant and other resources from the NC3Rs to ensure that our experiments are conducted in the most refined and effective way. We will also use the updated ARRIVE guidelines in our reporting.

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

We use the information in the NC3Rs website to inform us of best practices in terms of the 3Rs.

12. Gene-Environment 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
  - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants

Key words

Gene-environment interactions, Air pollution, Toxicity mechanisms, Biomarkers, Individual susceptibility

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 program of work aims to improve our understanding of the health consequences of exposure to environmental pollution in living organisms. We will use mouse models to study how environmental pollution affects cellular processes associated with toxicity and as a consequence the exacerbation 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?

Exposure to environmental pollution has been linked to numerous diseases and health conditions in humans. However, the exact mechanisms of toxicity remain to be elucidated. The mouse models we have developed allow the early detection of activation of cellular processes associated with toxicity prior to the induction of overt cellular damage. Additionally, the use of such models enables exploration of indirect mechanisms of toxicity, such as detecting cellular changes in the heart or brain following exposure in the lungs to an inhaled pollutant. Identifying the mechanisms of toxicity of environmental pollutants would facilitate the development of markers of early toxicity which would be of key importance in epidemiological studies. The data would also contribute to the early diagnosis and prevention of complex human disorders caused by such toxicity, for example neurodegenerative diseases.

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

Data will be generated characterising the deleterious cellular responses to a range of pollutants generated from different sources e.g. diesel exhaust, wood burning and cooking emissions, and help identify the deleterious constituents in a cellular and tissue-specific manner. For example, how this may impact neurodegenerative disease progression. In general the detection of cell and tissue specific responses will be undertaken on tissues harvested at the end of experiments using a range of approaches. However, blood samples may also be collected during the course of experiments which will then be analysed for serum biochemistry to detect changes over time. In particular, our genetically altered mice allow for the detection of a specific hormone in blood that is induced following cellular stress. A component of this project will establish whether specific vulnerable groups are more susceptible to pollutants. For example, those with inherited susceptibility to particular diseases, e.g. Alzheimer’s Disease. These results will be compiled into publications in peer-reviewed journals as well as presented at international conferences. In the future, this data has the potential to be used to identify biomarkers of early toxicity that can be used to develop preventative treatments. Through our collaborative network the data generated in this programme will be transmitted to government agencies and policy decision makers on the hazards associated with environmental exposure.

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

The data generated in this project will contribute to a mechanistic understanding of the health risks of different environmental pollutants and whether there are particular vulnerable groups. This work is part of a UK-wide multidisciplinary consortium funded by UKRI with specific goals to engage with government bodies. These data may be used to facilitate the development of government policies aimed at reducing environmental risk and be valuable to government and regulatory authorities. In the long term our work will contribute to the instigation of health interventions or government policies which improve human health.

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

Publications will be submitted to highly ranked peer-reviewed journals and where possible, data will be uploaded to relevant repositories. We will also present out work at scientific meetings and conferences. The animal models and any tissues will also be made available to other researchers. As part of our programme of work, together with our collaborators we will engage with government agencies to discuss the health implications of our studies.

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 used as they are a well characterised model that can be genetically altered to express early markers of toxic effects. Mice will principally be used for exposure experiments as adults to investigate the effects of exposure on developmentally mature animals. In addition we have evidence of transplacental and trans-lactational transfer of the effects of pollutants through generations. Some studies may involve analysis in foetuses, neonates and juveniles, such as to detect effects from transgenerational exposure and how this may change at various stages of development. As genetically altered models will be bred as part of this project, mice of all life stages will be needed.

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

Mice will be housed in standard cages for the duration of their lives. Mice used for environmental exposures will typically be exposed to a pollutant through injection, orally, or via inhalation, either as a single dose or repeated doses over time. Some substances will be administered with repeated doses, typically once daily for five consecutive days but will depend on route. The treatments are typically at a level not expected to induce any overt signs of stress. Where treatments are given over time, mice will be monitored closely for any signs of ill health. Some mice will be exposed using a nose-only inhalation system, which will require physically restraining the mice in a tube through a yoke on their shoulders. Mice will be humanely culled and tissue samples collected for analysis.

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

Typically, mice will only experience brief discomfort upon administering a substance. Where a noseonly inhalation exposure is used, mice will be restrained in tubes. This is expected to cause discomfort and stress, but no pain. Mice will be gradually acclimatised to the restraining tubes in order to reduce their stress. Any mice showing distress from restraint through signs such as vocalisation, flattened ears, or spiky coat will be immediately removed from the apparatus. This system of nose-only inhalation exposure and the yoke restraints has been successfully used in other institutions and advice on best practice for use and acclimatisation will be sought where possible.

With the exception of distress due to restraint for nose-only inhalation exposure, none of the protocols in this study are expected to cause more than transient pain, suffering, or distress, with dosing concentrations and regimes designed to not cause overt toxicity. Any clinical signs observed in mice at any point will be immediately flagged for consultation with Named Animal Care and Welfare Officer (NACWO) and/or Named Animal Veterinary Surgeon (NVS), with any prolonged clinical signs resulting in immediate removal from study.

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 genetically altered mice lines do not have any harmful phenotype. Mice used for exposures are typically expected to experience only brief discomfort except in the use of the nose-only inhalation exposures.

Overall, expected severities for the mice are 70% sub-threshold, 25% mild, and 5% 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?

Mechanisms of chemical and particle toxicity are complex and can involve multiple physiological pathways and multiple tissues. At the present time there are no alternatives to the use of animals to fully capture this physiological complexity. To exemplify this, we will study indirect mechanisms of response to environmental exposure. For example, if exposure to a pollutant is via the lungs, it has the potential to cause inflammatory changes in the lungs which could affect responses in other tissues such as the brain or heart. Ex vivo or in vitro experiments would only allow us to look at one organ system at a time and would not be able to detect any systemic responses.

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

We have considered using immortalised cell lines.

Why were they not suitable?

We will use immortalised cell lines for certain types of study. However, as detailed above in vivo physiological complexity which can determine chemical toxicity cannot be reproduced in cell culture. In addition, certain key determinates of chemical toxicity, for example pollutant metabolism, is not manifest in cell lines.

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?

Several colonies of genetically altered mice will need to be maintained throughout the course of the project. These genetically altered lines have been very well characterised and have a consistent response with minimal variation between individual mice, therefore typically 4 mice per group used for exposure experiments is sufficient to detect any changes. A control group is needed each time to allow for the detection of any outside influences but a positive control group is typically unnecessary. Data will be maximised by collecting a wide array of tissue types in each experiment. Where a novel compound is to be used, pilot experiments will be implemented to determine an appropriate dose and treatment regime, as well as to identify possible signs of overt toxicity caused by each compound.

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

Our well characterised mouse models allow for reduced sample sizes and negate the need for a control group known to have an effect. We will use both sexes and randomise mice to experimental groups. In some of our studies we will take blood samples at varying timepoints to detect changes in cellular stress responses over time within the same cohort of mice to reduce the need for replication.

Experiments will be carried out according to NC3Rs guidelines.

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 for breeding purposes will be minimised by careful selection and where possible experimental mice will be taken from maintenance breeding pairs. Mice have been generated from an inbred strain to minimise variability so they will maintain a stable genetic makeup. Part of this work will be collaborative with tissues derived from our studies sent to other institutes for further analysis.

We are engaged in a collaboration to develop in vitro chip-based assays to study mechanisms of chemical toxicity. For these studies we will generate embryonic stem cells from the reporter mice which can then be differentiated into different cell types e.g. hepatocytes (liver cells) and neurons (nerve cells), in vitro. These cells will be applied to microchips and the activity of the genetically altered toxicity markers used as an indicator of pathway activation. For these studies to generate embryonic stem cells reporter mice will be super-ovulated (drug induced release of multiple eggs that will then be collected once the mouse has been humanely killed).

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 well characterised genetically altered mice models which allow the early detection of cellular changes which pre-empt overt toxicity. This avoids generating overt toxicity in the mice which is normal practice in toxicological studies. The experimental protocols will involve administration of pollutants or specific chemical components of pollutants to the mice followed by the measurement of cellular changes in tissues obtained at necropsy. The dosing of the chemicals is not anticipated to cause pain or suffering or lasting harm. However, mice will be carefully monitored to ensure their discomfort does not go beyond moderate. If mice present with any clinical signs, steps will be taken immediately to reduce their discomfort, such as providing softened food and a warmed cage. If affected mice do not return to normal behaviour within a few hours they will be humanely killed. Where possible and where it does not conflict with experimental aims, we will endeavour to incorporate new methods of dosing including voluntary administration. However, this may not also be possible due to solubility of the drugs required routes of administration. In addition, as we typically use sub-toxic concentrations of compounds, we will need to ensure that there are not any confounding effects of other agents that could potentially nullify the study results. Where mice will be repeatedly handled, such as with repeated dosing or where they will be placed in a nose-only inhalation tube, mice will be handled via normal tube handling prior to the start of the study so that they can become acclimatised to handling and the researcher who will be performing the study.

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

There are major species differences in sensitivity to environmental toxins and although some fundamental mechanisms of cellular responses, for example those relating to ionising radiation, are conserved across species and can be studies in simple organisms the subtleties of pathways of chemical toxicity do not translate across species. As our aim is centred around investigating health consequences following environmental exposures, with a particular focus on cellular toxicity and its possible exacerbation of human diseases, we require a model with a high degree of translatability. That is, we need a model species that responds to environmental pollutants in a similar mechanism to humans, both in physiology with respect to tissue types affected and how these systems interact, as well as molecularly with similar genes and proteins involved that can be targeted and examined. In addition, the model species must have suitable disease models that can be used for our studies, such as those with increased susceptibility to neurodegenerative diseases including Alzheimer’s Disease, and must also be suitable for genetic manipulation for detection of markers of toxicity with a high homology to human systems. For these reasons, we require the use of mice and less sentient species are not suitable for this project.

The main goal of our study programme is to define pathways of toxicity in adult mice. Also, the pathways we are interested in may only develop over time. Some of the responses we will be monitoring in response to chemical exposure may only manifest over time, such as several weeks post exposure. To minimise variability we require a model that will not change in other ways during this time, that is, a developmentally mature model.

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

Drug doses will be carefully chosen to limit any overt toxicity effects. Treated mice will be carefully monitored including regular weighing and scoring sheets will be used where appropriate. Mice will be trained and acclimatised to the use of restraining tubes when used for nose-only inhalation exposures.

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

Guidance from NC3Rs and the PREPARE guidelines, as well as relevant EU guidelines, will be used where appropriate.

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

We will regularly check the NC3Rs website and attend meetings and symposia organised to discuss advances in the 3Rs. We will also keep a close eye on the development of novel experimental approaches which address the 3Rs from the scientific literature.

13. Studying liver disease and fibrosis

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

liver, fibrosis, therapy, wound healing

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?

The aim of this program of work is to advance our understanding of how liver disease and fibrosis (scar formation in damaged liver tissue) develops to help identify new drug targets and test new medicines for the treatment of these 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?

Liver disease is the third biggest cause of death of people of working age people in the UK, and the number of deaths are continuing to rise. In 2022 there was a 22% increase on hospital admission for chronic liver disease (CLD) on the previous year. This is ~47% higher than a decade ago. Up to 90% of CLD is caused by excess alcohol consumption, obesity/metabolic liver disease or viral infection (hepatitis).

Liver fibrosis, or scarring in the liver, is caused by repeated injury caused by CLD. Currently there are no clinically approved medicines to treat liver fibrosis. However, liver fibrosis is reversible if you remove or treat the underlying injury (e.g. anti-viral therapy), and the liver has a huge capacity to regenerate, therefore an effective medicine could reverse the disease.

It is therefore vital that we better understand the disease biology and identify novel drug targets.

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

This project license will generate new knowledge that will help advance our understanding of how scars form in the liver when it is damaged, a process called fibrosis, and why they persist during chronic liver disease. Our experiments will help us determine what changes at the level of molecules and cells when the disease gets worse, and if it improves e.g. with therapy. This work will help us to identify new drug targets, and test medicines which limit scar formation during liver injury and reduce liver fibrosis.

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

In the short-term, scientists in both the academic and pharmaceutical communities will benefit from the discoveries generated under this program of work. This could be due to the development of new research tools, experimental approaches or the identification of new pathways, which when targeted yield therapeutic benefit.

Ultimately, the long-term aim is to benefit patients through the development of new treatment strategies.

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

Our discoveries will be shared with the research community through presentations at national and international scientific meetings, and by publishing our data. We will also work with academics or the pharmaceutical industry, to maximise the impact of work.

We are committed to open science and wherever possible we collaborate with others to share tissue samples or cells, provide training in methods through collaborative research and participate in workshops to transfer any knowledge gained to the wider community.

Species and numbers of animals expected to be used

- Rats: up to 350

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.

Liver disease is a complex process that develops and resolves over many weeks and not only involves the liver as the affected organ, but also requires recruitment of immune cells. For this reason, it is not possible to fully study these events in cell or tissue slice cultures in the laboratory.

Liver disease and subsequently liver fibrosis develops over many years therefore adult rats will be used. Rats are used as the liver metabolism and immune system is close to that of humans.

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

Chemical and dietary models of liver fibrosis have been refined for many years and are very predictable models of CLD, therefore we know what disease stage animals have reached at any given time point. We have good experience running drug studies in these models, therefore we know exactly when to give drugs and for how long to administer them. For studies testing an anti-inflammatory or anti-fibrotic therapy, drugs will be administered either prophylactically (as disease develops) or therapeutically (once disease is established). In the liver disease model rats receive up to bi-weekly injections of the chemical into their belly for a maximum of 16 weeks or are fed a high-fat or high sugar diet (either with or without sugar water, comparable to coke), for up to 52 weeks to cause liver injury and liver fibrosis. In acute models of chemical injury, to study inflammation and wound repair in the liver, rats receive only a single injection of the chemical into their belly.

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

In our liver injury models, rats may show signs of sickness e.g. hunched posture, diarrhoea, ruffled fur, look pale or feel cold. A range of supportive care and pain relief will be given and if the general clinical condition does not improve within a maximum of 24h the animal 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)?

Cell isolation from liver tissue in non-recovery procedures comprise ~30% of experiments, whilst mild models of disease such as acute liver injury will likely make up 20% of studies and moderate chemically-induced or dietary-induced models of liver disease and fibrosis, either with or without a therapy will comprise ~50% of the work.

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?

Liver disease is complex and develops and heals over many years. Liver fibrosis is a term which describes scar formation in the liver because of repeated tissue damage. In response to injury, many different cells in the liver change their behaviour to deposit scar tissue to try and heal the wound. If the damage is only small or limited to one insult, then the scar will dissolve. However, if the liver is repeatedly damaged, the scar tissue persists, which can reduce the ability of the organ to perform normal daily tasks. When disease is very advanced the liver can fail. Fibrosis occurs not only as a consequence of damage to the liver but also through recruitment of white blood cells to the injured tissue, therefore signals from outside the liver are also an important part of the disease process.

For these reasons we need to perform some of our research and drug testing in animals.

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

Human tissue samples: Wherever possible, we will use human tissue or cell line culture systems to replace animal models of liver disease. We regularly isolate liver cells e.g. hepatic myofibroblasts (scar forming cells) and hepatocytes (functional cells which comprise 80% of cells in the liver), from the margins of resections of human liver tissues, and are testing/developing 3D cell culture models such as human Precision Cut Liver Slice (hPCLS) cultures an alternative methods for drug screening and to understand the biology of scar cells. These 3D culture models will not only reduce animal use but help identify compounds which are toxic and therefore not be used in vivo, or help refine the dosing regime for in vivo studies to reduce the risk of unwanted toxicity.

Use of 3D models and tissue slices has also led to a direct reduction in the number of rats required to prepare individual cell populations and to run disease models.

We regularly search the literature and use the internet (google searches) or pubmed (a database of scientific papers), attend scientific meetings, or use websites such as the National Centre for Replacement, Reduction and Refinement (NC3Rs) to find new non-animal alternative methods and approaches to implement in our research.

Why were they not suitable?

2D and 3D cell culture (e.g. spheroids or organoids which are 3 dimensional balls/aggregates of cells) models of primary cells or cell lines are useful tools to help understand the biology of that cell type and screen drugs for efficacy e.g. promote cell death or limit the fibrotic phenotype of scar forming hepatic myofibroblasts (HM).

However, culturing HM on stiff culture plastic is artificial and the mechanical stress of tissue culture plastic has been shown to drastically alter the phenotype of the cells, where the HM adopts a “super activated” and produce huge amounts of scar-promoting factors, thereby increasing the chance of seeing false positives or false negatives in a drug screen. Similarly, hepatocytes rapidly change their biology in culture and within 24 hours, lose expression of key proteins which perform daily liver functions such as the breakdown drugs or fats and remove toxins from the blood.

Liver fibrosis development and resolution is regulated by many different types of cells communicating with each other within the damaged organ as well as through communication with white blood cells and receiving signals from other organs. Recreating all of these inter and intra- organ damage signals is difficult to model in culture, even in tissue slice 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 have used knowledge from previous studies and use this 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.

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

We have taken multiple approaches to ensure that we use the minimum number of animals for research purposes. For example, we have performed mathematical calculations called power calculations to predict the minimum number of animals needed for an experiment. We have also drawn our experience, looked at published data and used online experimental design tools e.g. NC3R's Experimental Design Assistant .

We have performed audits of our previous research studies and assessed research plans of current projects to predict use under this project.

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 need to generate biologically meaningful data. If this is not possible then pilot studies are performed to reduce numbers and inform future studies going forward.

Tissue sharing with other research teams or through internal/external collaborations and accessing tissue from our archives or via external sample repositories are aways considered as an alternative source of samples, to limit 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.

All of the disease models chosen are the lowest severity model that can be used to answer our research questions. Accumulation of scar tissue , namely fibrosis, in the liver is caused by many different types of injury. Therefore to understand how this disease develops, and test new therapies, we need to perform the models in animals.

Chemical models of liver fibrosis have been refined for many years and is a very predictable model, therefore we can predict the disease stage at any given time point.

To model diet induced liver disease we will feed rats a modified diet (e.g. high fat) either with or without sugar water (comparable to coke). These animals gain weight and develop features of fat induced liver disease.

Therapies are given using the most refined method/route and frequency for the nature of the drug, compound or therapeutic molecule being given. Blood sampling and glucose tolerance tests are performed using the most refined method.

These are currently the most refined models, but we will always aim to implement any future refinements which emerge.

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

The liver contains lots of different types of cells and these cells all talk to each other to ensure that the liver does its job properly. When the liver is damaged, all of the different cell types are needed to help heal the injury. White blood cells also enter the liver at the site of injury to help clean up the damage and heal the wound. However, when the liver is repeatedly damaged this healing process becomes abnormal and fibrosis occurs. Because of the complex nature of the disease process it is not possible to recreate this using cells in culture dishes. For this reason we need to study disease progression and test medicines in the whole animal.

Liver fibrosis is an inflammatory driven disease. 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 or type of therapy tested. 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 and pain relief is readily provided to minimise distress or suffering if this occurs and improve animal welfare.

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. The PREPARE Guidelines on the Norecopa website and the Experimental Design Assistant (EDA) tool on the NC3Rs website, will be used to ensure experiments are conducted in the most refined way.

The General principles for welfare assessments and guidance outlined on the NC3Rs website https://www.nc3rs.org.uk/3rs-resources/welfare-assessment will be used throughout the project to help inform if any animal is suffering distress and refine humane endpoints.

Should distress occur immediate actions as described in the individual protocols would be taken to reduce an animals suffering.

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

There are many sources 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 nonanimal 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.

14. Development and use of molecular imaging tools for cardiovascular applications

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

cardiovascular, imaging, radiotherapy, therapy, physiology

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 aim is to develop new imaging agents (or new imaging techniques) for imaging the heart and cardiovascular system.

These imaging tools could be used for more sensitive or more informative detection of heart disease in humans, to better understand cardiovascular biology in animals and humans, or to help develop or test new drugs for the treatment of heart disease.

To develop these imaging tools it may be necessary to create, adapt or improve animal models of heart disease to make them as relevant to human diseases as possible to ensure that our imaging agents have the best chance of working when we then advance them to testing in 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?

Heart and circulatory diseases remain the most common cause of death in the UK and are responsible for approximately 240,000 deaths per year. Coronary heart disease is the most common single cause of death affecting about 117,000 people per year. At present, many types of heart disease are diagnosed by measuring changes in the dimensions of the heart or changes in the way that it contracts using imaging techniques like echocardiography using ultrasound or magnetic resonance imaging (MRI). However, these techniques are very insensitive, and by the time heart disease is severe enough to be measured by them, treatment options for the patient can be very limited. For example, approximately half of patients affected by a heart attack die immediately, often before reaching hospital. While their death is sudden, the disease which led to the heart attack has often been progressing undiagnosed beforehand for many years. Similarly, many cancer treatments can have major side-effects, particularly for the heart. This “cardiotoxicity” can take years or even decades to become severe enough to be detected by echocardiography or MRI, but once these techniques do eventually detect the problem, the disease can be so advanced that the only option for a patent can be a heart transplant.

The purpose of this project is to develop novel "molecular" imaging techniques which can help detect early changes in the biochemistry of the heart before it changes in shape, size, or the way that it contracts. These imaging tools could help us detect heart disease sooner, or help us better understand what’s going wrong in a patient’s heart so that we can better personalise their treatment. Because these biochemical changes tend to happen before contractile changes, our imaging tools could also be used to provide feedback on how well or how accurately new drugs are hitting their targets, which could speed up drug development and make it cheaper, and possibly require fewer animals to be used in testing.

To develop these molecular imaging agents we need to understand how they behave in the body, how accurately they can detect and monitor heart disease, and how well they report on the effectiveness of new heart-protecting drugs. Unfortunately, these questions can only be addressed using experimental models of these diseases in animal models.

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

This project will allow us, with the help of animal models, to develop new imaging techniques to better understand and diagnose evolving cardiovascular disease, and to better understand the nature of cardiovascular biology in health and disease.

These imaging tools would allow us to treat cardiac patients more effectively and monitor them during and after their treatment. They will allow us to identify patients at risk of cardiovascular disease sooner, to better distinguish different types or heart disease to make sure patients get the most accurate diagnosis and treatment, and to monitor patients during and after their treatment to ensure that treatments are working. Our imaging techniques will also provide early readouts of how well new heart drugs are working to speed up the development process and make it cheaper.

Ultimately this project aims to reduce the incidence and severity of cardiovascular disease by early detection and earlier and better targeted treatment. It will produce multiple outputs

including new imaging agents, better understanding of cardiovascular biology in health and disease, and provide a new platform for developing drugs to protect the heart. Much of this work would result in new publications.

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

Short term (during the project): to inform decision making on whether to test new imaging agents in humans, or return to the laboratory to improve them further, or abandon them and investigate different design approaches.

Outputs - new imaging agents, new imaging techniques, refined animal models for testing our imaging agents including models most closely replicating human diseases, new publications demonstrating proof-of-concept for future more detailed studies and applications. Beneficiaries: our research team and our close collaborators.

Intermediate term (during the project and within a few years thereafter): Imaging agents validated in clinically relevant animal models. Proof of concept for the use of our imaging tools in drug development. Data to support applications to produce new imaging agents in small scale clinical trials. New insights into cardiovascular biology and cardiovascular disease which may spawn new ideas for further new imaging agents or drugs. Opportunities for the application of our imaging tools outside their original purpose (e.g. in other diseases).

Outputs: new imaging agents ready for production to Good Manufacturing Practice (GMP) standards and early proof of concept clinical trials. Application of imaging agents to answer new biological questions and obtain new insight into disease processes (and higher impact publications).

Beneficiaries: our team and collaborators, new collaborators from other disciplines, research tools for both the imaging and cardiovascular research communities more widely.

Long term (after the project): Larger scale clinical trials, validated clinically useful tools for detecting cardiovascular disease earlier and/or more accurately, a more widely available platform for developing new drugs outside the initial scope of this project. Outputs: New licenced imaging agents and cardioprotective drugs or strategies, leading to better patient management and clinical decision making (highest impact publications). Beneficiaries: patients, other research scientists in the field, scientists in other disciplines, health services and pharmaceutical companies.

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

We will continue to build our collaborative networks spanning synthetic radiochemistry to basic biology to clinical cardiology and imaging science. We will collaborate with industrial partners to ensure that our imaging agents can be tested in phase II and III studies for safety and efficacy and ultimately be commercialised to ensure widespread clinical use. We will disseminate our results at conferences and workshops and publish in peer reviewed national and international scientific journals. We will organise workshops at London-wide or national events when warranted to share our results and provide handson training for other scientists interested in this work. Other means of dissemination will include talks at the Pint of Science initiative or active participation in the summer festival of the Royal Society. of dissemination will include talks at the Pint of Science initiative or active participation in the summer festival of the Royal Society.

Species and numbers of animals expected to be used

- Mice: 1500
- Rats: 1450

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 mouse and rat models of cardiovascular diseases are used in this project license because they reproduce many of the widely reported biochemical and physiological hallmarks associated with the development of cardiac hypertrophy, heart failure, cardiotoxicity, and vascular changes known to exist in their corresponding human conditions.

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

Typically, an animal in this Project License will:

Undergo baseline imaging scan(s) with blood sampling to establish baseline measurements for that animal.

Be induced to express cardiovascular disease including cardiac hypertrophy, heart failure, coronary microvascular disease, cardiotoxicity and vascular remodeling either surgically, pharmacologically, through diet or by external beam radiation therapy.

Be imaged in vivo by ultrasound, X-ray computed tomography (CT), magnetic resonance imaging

(MRI), positron emission tomography (PET), single photon emission computed tomography (SPECT),

(intravascular ultrasound) (IVUS) under either inhalable or injectable anaesthesia to detect cardiovascular diseases with or without administration of imaging agents.

Be treated with agents to modulate or prevent cardiovascular diseases (either before, during or after the induction of disease).

Be humanely culled.

The duration of the experiment will depend on the aim of the study and the individual protocol used. However, all protocols have been refined to the minimum experimental duration necessary to achieve the scientific objectives. Most protocols will be shorter than 3 months, but for clinically relevant models of slow-evolving cardiovascular disease (particularly when induced by external beam radiation) it may be necessary for experiments to run for up to one year.

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

Induction of cardiovascular diseases including cardiotoxicity, cardiac hypertrophy, and vascular remodelling. On average, most cardiac hypertrophy and pharmacologically-induced cardiotoxicity protocols would not exceed 12 weeks in duration, while external

beam radiation-induced protocols may last for up to 1 year because the rate of development of cardiovascular disease is much slower.

Changes to general wellbeing: induction of cardiovascular disease may lead to changes in an animal's physical characteristics (weight, coat condition, diarrhoea, and breathing) and behaviours (posture, levels of activity, peer interaction) and will be monitored with this possibility in mind.

Risk of pain or infection following surgical procedures. All animals are expected to make a rapid and unremarkable recovery from the anaesthetic within two hours. Peri- and post-operative analgesia and antibiotics will be used to mitigate this as required. Uncommonly animals that fail to respond to these treatments or exhibit signs of pain, distress or of significant ill health will be humanely killed by a Schedule 1 method unless a programme of enhanced monitoring and care is instituted until the animal fully recovers.

Cumulative effects of repeated anaesthesia from repeated imaging studies. All animals are expected to make a rapid and unremarkable recovery from the anaesthetic within two hours.

Uncommonly animals that fail to do so or exhibit signs of pain, distress or of significant ill health will be humanely killed by a Schedule 1 method unless a programme of enhanced monitoring and care is instituted until the animal fully recovers.

Cardio-respiratory distress (rapid breathing, tachycardia, wheezing) would constitute an end pointand result in immediate humane culling

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:

Non recovery: 17% mild: 8% moderate: 75%

Rats:

Non recovery: 52% mild: 5% moderate: 43%

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?

Regulatory agencies require animal data to demonstrate safety and effectiveness before molecular imaging agents or drugs can enter human trials.

We need to prove that our molecular imaging approaches work, and that they accurately report on the biochemical processes that we have designed them to do. This means that we may have to perform experiments where (for example) we use drugs or treatments to change the biochemistry of the disease process and show that our imaging agents can respond to that change. Experiments like this cannot be conducted in humans for ethical and scientific reasons.

We need to develop and optimise our imaging agents in living organisms which have similar biology and biochemistry to humans. This includes the need for animal models where imaging agents are absorbed, metabolised and excreted in the same way as they would in humans. This is not possible with simpler experimental systems like cell culture or computer modelling.

Most aspects of cardiovascular pathologies can only be studied in live animals (e.g. atherosclerotic plaque progression, the development of cardiotoxicity, high blood pressure (hypertension), thickening of the heart wall (hypertrophy), or death of heart tissue (myocardial infarction) because there are complex interactions between different body systems which cannot be replicated in anything other than an intact animal.

Our imaging agents are designed to detect the earliest stages of disease, often long before a patient would even know they are sick or are showing any clinical symptoms. It would be impossible to recruit such patients for human studies for this purpose. The only way of studying (or developing imaging agents for) these earliest stages of disease is to use animal models where we know when we have induced a disease process (and can do so reproducibly) and can follow the biological changes over time.

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

We have considered the following alternatives:

In silico/computational methods where computer simulations and data analysis are used to predict the behaviour of biological systems or how an imaging agent might behave in cells or the body.

Cell culture methods where cells from relevant tissues are grown in a controlled environment outside of an organism. They can be used to confirm that an imaging agent gets taken up in their target cells or respond to a specific intracellular process. Cell culture techniques can also be used to determine how an imaging agent gets trapped within a cell, or whether it gets metabolized and what it might get broken down to, whether an imaging agent is toxic to cells.

First in man studies, where experimental drugs or imaging agents are administered to human participants without prior animal testing.

Why were they not suitable?

In silico/computational methods are not yet able to accurately predict how an imaging agent or drug will behave in the body in terms of its stability, vulnerability to metabolism, its specificity for its target compared to no-target cells, tissues and organs, its pharmacokinetics and likely routes of clearance etc. For such in silico models to be used they require animal data to build the machine learning models in the first place, so they can still not be considered true “alternatives”.

Cell-based methods are not able to accurately predict how an imaging agent may behave in intact tissues or in the body, or (for example) how an imaging agent might traverse blood

vessels to target biochemical processes in the underlying tissues, or how well they wash out from normal tissues so that we can only see the diseased cells of interest inside the body. Cell culture models can not accurately recapitulate complex 3-dimensional multi-organ disease processes which are essential for testing and developing our imaging agents.

First in humans: Most of the imaging agents to be studied have not been used in humans before and require animal data before regulatory approval. In many cases, it is unethical to use experimental tools (such as inhibitor drugs) in humans to confirm that our imaging agents are specifically hitting their biological target. In many cases the imaging agents we plan to develop target the earliest stages of disease before symptoms are measurable. It would therefore not be possible to identify and recruit patients at such early stages of disease. Even in patients who are exhibiting clinical symptoms there are many variations between people in terms of age, sex, ethnicity, diet, lifestyle, other co-existing diseases etc. which may affect the behaviour of our imaging agents, make it very difficult to interpret the variable readouts that our imaging techniques provide. We need to first prove that our imaging agents are specific to a specific biochemical process in animal models which have much less variability between individuals, and where we can prove that they can image a disease process without these confounding factors. Animal models also allow us to study the earliest stages of disease because we can image the disease process from the moment we induce it.

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 experimental group sizes are estimated based on the typical degrees of variation between individuals we have observed in previous experiments, or from previous relevant literature. For new imaging agents or the testing of existing imaging agents in new or refined animal models, we use pilot experiments to generate data that we then use to calculate the number of animals we would need in larger studies to prove a statistical effect. Generally we find that our imaging agents provide very reproducible readouts of biochemical processes which can allow us to use smaller numbers of animals in our experiments.

The total numbers of animals that we have estimated for the proposed 5-year period are based on a combination of a projection of annual returns data from the past 5 years, the number of staff we anticipate within our group who are likely to be working on this PPL, and how many studies this may enable. That may vary widely between protocols according to how long each needs to run. For example, isolated perfused heart experiments are very fast to perform (on average one hour per experiment), so that several can be done in a day. Conversely our experiments aiming to detect radiation-induced cardiotoxicity may need to run for nearly a year because the injury takes a very long time to develop. This means that we are limited to lower animal numbers for these long studies because of the considerable costs involved, both in imaging and animal maintenance.

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 where necessary on small cohorts, to provide statistical data allowing animal number estimations for definitive larger experiments.

Wherever possible, we will use more than one imaging approach on the same animals so that we can study two or more biochemical processes in the same animal at the same time, or be able to relate our imaging agent performance to more traditional imaging approaches (such as ultrasound) to demonstrate that our molecular imaging agents are more sensitive or can detect disease earlier in the pathology. Using more than one readout at the same time allows us to halve (or more) the number of animals that we use. To further reduce the number of animals we use, we are increasingly comparing imaging readouts directly with other biochemical measurements of disease obtained post-mortem on an individual-by-individual basis, rather than just comparing the averages across whole groups using a variety of statistical approaches (such as correlative analysis, principal component analysis or nonhierarchical analysis, as exemplified in this recent publication https://www.biorxiv.org/content/10.1101/2024.04.22.590587v1). If there are wide ranges in terms of the rate of development or the severity of disease, this quite often requires an increase in group size to ensure that we can statistically detect differences. These statistical approaches allow us to account for these inter-individual differences without necessarily increasing animal cohort sizes.

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

Molecular imaging technologies offer several key opportunities to reduce animal numbers. Because they allow repeated measurements on the same animal over time, animals are only killed at the last time-point. If for example we needed to study disease at 8 time points with a required group size of 6 animals (plus parallel time-matched control animals), we would require (8 x 6) x 2 = 96 animals. However, by using imaging we can follow the same biochemical processes non-invasively in the same animals, and only require 6 x 2 = 12 animals. Since each animal can also be used as its own control, inter-individual differences can be corrected for in our analysis, offering the possibility of needing fewer animals per group to detect a statistically significant effect.

Moreover, not only imaging agent distribution in vivo, but potential time-dependent and unexpected redistribution can be detected through serial imaging. All these attributes contribute to a greatly improved benefit: cost ratio (benefit=data quality/quantity, cost=animal numbers/procedures). Recent refinements include the parallel measurement of multiple post-mortem biochemical biomarkers of disease, which are correlated on an animal-by-animal basis with our imaging agent uptake or pharmacokinetics using a combination of statistical methods including Principal Component Analysis. This approach enables us to exploit the inter-individual variability between animals in our experimental groups to better validate the sensitivity of our imaging agents, meaning that variability between animals becomes a benefit to our approaches, which can potentially be harnessed to reduce animal numbers required for a study.

Where possible we will share tissue from our animal models for other potential applications. A good example of this is a current study where we are focusing on imaging the mitochondria in the heart as an early indicator of cancer therapy-related cardiac dysfunction. In these studies we are also going to include the imaging and collection of skeletal muscle (from both control and treated animals) to share with a collaborator whose

research focuses on skeletal muscle metabolism in health and disease. There are many instances where cardiovascular disease leads to disease in other organs, and vice

versa. Where appropriate we will make those tissues and imaging datasets available to other groups for hypothesis building and pilot data.

Using pilot studies coupled with imaging allows us to use far fewer animals in determining the best imaging agent candidates to progress and to design the optimum definitive experimental study going forward. Where statistically appropriate, data from pilot studies will be expanded to form larger experimental cohorts in the main study to reduce 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 ethos throughout this project is to create, validate and use imaging agents capable of providing the richest, most sensitive and earliest non-invasive biomarkers of evolving disease. Our primary ambition is to develop imaging agents capable of detecting and characterising cardiovascular disease before it is detectable by standard clinical imaging techniques like echocardiography, which itself commonly occurs long before clinical symptoms manifest.

Wherever possible we will obtain as much information as possible about the pharmacokinetics, selectivity and sensitivity of our new imaging agents by first screening and validating them using our isolated perfused heart models. As a model obtained through terminal anaesthesia, this causes our rodents the least pain, suffering and distress. Only once screened and successfully validated using this model would our candidate imaging agents then be tested in in vivo models of cardiovascular disease. This approach may not always be possible however, it may be that we cannot accurately model the disease, or the chemistry of the imaging agent may be too slow to react in the heart fast enough to build up to detectable levels so that we can measure it. In instances like this we would bypass this step and move directly to in vivo models instead.

Our in vivo models typically employ a tiered approach. First, we obtain proof of concept that our imaging agent successfully targets a specific disease process in an acute model where we know that our biochemical target is evident and/or abundant. To validate the mechanism of targeting we may include experiments which change the abundance of that target, and in turn the response of our imaging agent. Having validated our imaging agent in this way we would then progress to more refined models which are closer in severity and timeline to the condition that we ultimately intend to image in humans. In this way we limit the number of animals that we subject to chronic diseases which may cause prolonged suffering or distress while still ensuring that our imaging agents have the greatest chance of successfully translating to clinical use.

For our models of cardiotoxicity we would initially use higher doses of chemotherapy agents or whole heart irradiation to invoke a more extreme or rapidly evolving cardiotoxicity to confirm that our imaging agents can hit their biological targets (and confirm that those targets exist in those hearts by postmortem biochemical assays or histology). Once validated we would then progress to more clinically relevant lower doses of chemotherapeutic agents, or lower or more geographically targeted radiotherapy protocols which express those imaging targets in lower concentrations or over a more extended timeline.

Using the same rationale for our models of cardiac hypertrophy, in our previous work we have initially used surgical models like abdominal aortic constriction to cause cardiac hypertrophy within a few weeks, but have now refined this approach by infusing drugs like angiotensin II which cause a more titratable and slower evolution of hypertrophy over a longer timeline without the need for surgery beyond the implantation of subcutaneous minipumps, for example.

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

Mice and rats are the species of least neurophysiological sensitivity that exhibit comparable cardiovascular diseases to humans (e.g., cardiac hypertrophy, anthracycline cardiotoxicity, coronary microvascular disease), while also being large enough to accurately image them with the spatial resolution that our scanners are capable of (the resolution of our small animal scanners are in the order of 0.1-1mm).

Rats are often our preferred species because the larger amount of heart tissue that we can obtain from them (1g compared to 200mg for a mouse heart) means that we can perform more biochemical analyses from each heart to compare with our imaging agents, which allows us to better validate our imaging agents against more biomarkers of injury with fewer animals.

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

For the early evaluation of radiotracer selectivity, specificity and first-pass cardiac pharmacokinetics, we use an isolated perfused heart model using a unique gamma-detection apparatus that we have developed. This model is highly controllable and extremely reproducible and provides us with a useful initial screen and means of validating new imaging agents before progressing to animal models of disease.

As an intermediate step before progressing to animal models of disease we would first evaluate our imaging agents in naiive healthy animals to confirm that they do not accumulate in healthy tissues using authority from another project licence held within our department, and that their pharmacokinetic properties suggest that they would be capable of detecting disease if it were present (by establishing where the tracer normally goes in the body, how quickly it clears from blood and healthy tissues etc.). This level of screening allows initial testing of novel agents prior to disease induction.

Animals will be monitored daily post-surgical interventions and at least three times weekly once early clinical signs have developed. We routinely use monitoring scoring sheets to objectively record animal health indicators and weights. These help us to pick up any adverse events as early as possible, allowing us to either bring the experimental endpoint forward, obtain veterinary or welfare advice or cull the animal as appropriate.

Inhalation anaesthesia will be used wherever possible to minimise transient pain and distress during imaging. Full recovery between periods of anaesthesia, rehydration during long imaging sessions, respiration/cardiac function monitoring, body temperature monitoring/maintenance will be conducive to animal wellbeing. When novel compounds or dosages are being studied, small pilot experiments will help refine the experimental conditions prior to a larger more definitive experiment. Prior to novel substance use, the NVS will be consulted where appropriate, and animals will undergo more frequent monitoring post administration to minimise any welfare impacts.

The use of imaging biomarkers to detect and track disease will help us in many cases to better assess the overall disease burden in our animals. Wherever possible we will attempt to multiplex imaging agents to minimise the number of animals used, while also employing longitudinal imaging, which significantly reduces numbers by using animals as their own controls and add significantly to statistical power.

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

We will follow established published guidelines to ensure experiments are conducted in the most refined way. These include:

The Responsibility in the use of animals in bioscience research produced by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs).

The Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986.

The ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) are a checklist of recommendations to improve the reporting of research involving animals – maximising the quality and reliability of published research, and enabling others to better scrutinise, evaluate and reproduce it.

Accepted limits of volumes and frequencies when administrating compounds and anaesthesia (Appendix 1a in Action Plan section).

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

We will stay informed by updates from the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) website and seminars on the 3Rs organised within and outside of our institution.

Additionally, we have direct support and contact with NC3R's Programme Managers who supports the application of the 3Rs within our institution. Our institution has an internal 3Rs sub-group and a 3Rs Champion to promote advances in this field to all users. These resources provide expert advice and coordinate the sharing of best practice.

15. Identifying the factors affecting diadromous and freshwater fish populations

Project duration

5 years 0 months

Project purpose

- Protection of the natural environment in the interests of the health or welfare of man oranimals
- Research aimed at preserving the species of animal subjected to regulated procedures aspart of the programme of work

Key words

Fish movements, Fish ecology, Freshwater and marine environments, Anthropogenic effects, Fish conservation

Animal types	Life stages
Brown Trout (Salmo Trutta)	Juvenile, Adult, Pregnant adult, Aged animal
Salmon (Salmo salar)	Juvenile, Adult, Pregnant adult, Aged animal
All other fish	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 the project is to improve knowledge and understanding of the movements, migrations, patterns of distribution and behaviour of diadromous and freshwater fish populations, in relation to their environment and in particular to anthropogenic pressures. The knowledge and understanding will be used to provide evidence-based advice in support of management and conservation to stakeholders, 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?

Effective management of freshwater and diadromous fish populations requires reliable information (i.e. supported by research-based evidence) on the status of stocks, their ecology and movements, and the range of natural and anthropogenic factors (e.g. climate change, artificial light at night, water abstraction, fish stocking and non-native species) that can impact fish at different stages of their life cycles. The research proposed under this Project Licence will address aspects of these research priorities, with the ultimate aim of providing evidence-based management and conservation advice to support national government and international bodies which deliver policy relating to management and conservation of fish stocks.

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

The outputs of the work will include:

Data: individual-based data on migration routes, residence times and residence locations, preferred habitats and patterns of behaviour, including in response to environmental and anthropogenic factors;

Information and knowledge: improved understanding, enabling us to provide better advice on conservation and management, on topics such as monitoring methods, life history strategies and the individual and population responses to environmental drivers;

Publications: analysis of the data and knowledge gained for peer-reviewed 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 be achieved through advice and measures applied to specific study sites, for example by informing advice about mitigating the effects of in-river obstructions on migrating fish. In the medium and longer term, depending on the extent to which the new knowledge is species- or region-specific, the benefits could be achieved nationally and internationally and help to meet national and international commitments to fisheries or environmental management. The data obtained will help

to meet national or regional statutory obligations for rational and sustainable fisheries and environmental conservation. 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 external project partners. Significant new knowledge will be published in peer-reviewed journals or as publicly available reports, with a Communications Team supporting the dissemination of results and major programme highlights through traditional and social media outlets.

Species and numbers of animals expected to be used

- Brown Trout (Salmo Trutta): 18,750

- Salmon (Salmo salar): 18,750

Other fish:

- All other fish: 21,250

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.

The aim of the work is to advance our understanding of the movements and behaviour of diadromous and freshwater fishes, and to provide direct empirical evidence of responses to environmental or anthropogenic drivers. Much of the work under this licence will focus on species which have been selected as being of high conservation interest (including Atlantic salmon, brown trout, and European eel), as it is these species for which our work can provide greatest benefits for conservation and management. The life stages used will be selected to answer specific research questions, but in the case of Atlantic salmon and brown trout, many of the knowledge gaps relate to the ecology of juvenile fish and consequently research would be directed at that life stage, whilst ensuring that individuals are large enough to be tagged without adverse effects.

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

An animal used in this project will typically be captured using electric fishing or in a net or trap (with the most appropriate method selected to minimise adverse effects). Once caught, the fitness and wellbeing of the fish will be assessed to ensure that it is fit to undergo the protocols. The fish will then typically be transferred to a holding tank containing an anaesthetic, and then maintained under sedation or anaesthesia while protocols are conducted. This will typically involve the fish being measured and weighed. Optionally, a small number of scales may be collected (to enable ageing, or for genetic or isotope studies), and/or a small amount of tissue (a fin clip) or mucus may be collected (typically for genetic analysis). A small blood sample may optionally also be taken at this point. The fish would then typically be tagged, with the tag type dependent on the study question. Some tag types, particularly electronic tags, need to be placed inside the coelom and therefore require surgery, whereas others such as Passive Integrated Transponders require a less invasive process. Fish will then be placed in a recovery tank under observation to assess their fitness for release, at which point they will be discharged from the Act and returned to the wild.

A smaller number of fish may also be subjected to an additional protocol, stomach or colonic flushing, to gain data on their diet. These fish would also be captured from the wild and anaesthetised, and may also undergo biological sampling prior to the flushing, which involves the insertion of a tube into the stomach and flushing out of stomach/colon contents using distilled water or saline solution. The fish would then be allowed to recover, and after passing an assessment for fitness they would be released to the wild.

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

The procedures are assessed as being of Mild or Moderate severity. Possible adverse effects include:

Pain or distress during the tagging or sampling procedure. If necessary and beneficial, as for example during surgical tagging, anaesthesia or sedation will be used to minimise this risk. Whether or not anaesthesia and/or analgesia is used, fish will be handled carefully to minimise handling stress, and kept out of water for the minimum time necessary to undertake the procedure. Careful monitoring by experienced operatives will take place throughout the procedure, and until the fish is fully recovered and released.

Infection of tagging wounds or skin (as a result of handling or capture damage). Risk of infection will be minimised by creating aseptic conditions for tagging.

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 to minimise impact to the fish 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 postprocedural 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)?

The expected severities of the Protocols are 'Mild' and 'Moderate'.

In work on salmon and brown trout, 96% of animals under this Project Licence would experience 'Mild' severity, and 4% would experience 'Moderate' severity.

In work on other fish species, 96.8% of animals under this Project Licence would experience 'Mild' severity, and 3.2% would experience 'Moderate' severity.

What will happen to animals used in this project?

- Set free
- 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 principal aim of the work is to describe the ecology and behaviour of wild fish in relation to changes in their aquatic environment, to support their conservation and management. Therefore, there is no alternative to the use of living animals.

The data which will be gained from this project will fill knowledge gaps which hinder our ability to effectively manage these species, and will typically be species-specific (which drives our selection of particular species to work on).

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

Procedures will only be undertaken for fish species where gaps in knowledge cannot be filled with nonanimal alternatives. Work will only be initiated after a review of literature to

ensure that this project is not duplicating previous studies which can already provide the required data.

Environmental DNA sampling is an emerging new tool which can be used as a non-invasive method for determining presence or absence of fish in water bodies, and we will use this method where appropriate. However, it cannot provide the detail on fish biology, movements and habitat use for which the Protocols in this Project are better suited.

Why were they not suitable?

To monitor the movements and behaviour of diadromous and freshwater fishes to the required level of detail for conservation management, there is no viable alternative to tagging protocols. Similarly, stomach flushing is the only effective way to achieve data on fish diets from living fish.

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 estimate for numbers of animals used in each protocol are based on previous research experience and but will be re-assessed prior to any project being implemented (using support from in-house statisticians and information from peer-reviewed scientific literature). All experimental work will use advice from professional statisticians to ensure that the minimum number of animals are used that will permit a robust and meaningful statistical analysis of the results. These statisticians will provide statistical support to all aspects of the research, from designing the experimental approach to conducting and reporting the analyses.

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 (including the use of statistical power analysis to assess appropriate numbers for the study aims). As part of our internal 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.

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 Atlantic salmon, brown trout, European eel and other diadromous and freshwater species need to be studied in order to provide relevant and meaningful data to support policy and management decisions. The animal models and methods chosen will be those deemed most likely to provide valuable evidence to support conservation and management advice, based on previous experience and research (by ourselves and by other researchers). No endangered species, as defined in Annex A of Council Regulation 338/97, will be used on this project.

Best practice methods in fish capture, handling and regulated procedures will be followed to ensure that we minimise animal suffering. Where fish undergo a procedure and recovery, they will be monitored for a suitable period of time in order to assess any adverse impacts and ensure a minimum of suffering.

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

The aim of the work, to advance our understanding of the movements and behaviour of diadromous or freshwater fish in their natural environment, inherently precludes the use of terminally anaesthetised animals or less sentient species. The research questions addressed by this project would be specific to both species and life stages, which will determine the choice of study animal.

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

Where appropriate, anaesthesia and 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 (in addition to an assessment prior to starting the procedure).

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 continually improving effective methods. Tag attachment/implantation methods will be continually updated, and reviewed alongside tag technology developments to ensure that they are humane and that they minimise the effects on the fish’s behaviour, welfare and survival. Published studies on refinement of techniques will be used to help refine protocols and methods. Personal Licence holders and the Project Licence holder 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?

The Project Licence holder will stay informed about advances via continual review of peer-reviewed literature and regular discussion with the AWERB team. Literature review will focus on advances in replacement (e.g. use of non-invasive technologies such as environmental DNA sampling), reduction (e.g. looking at numbers of fish used in any similar studies, or looking for new studies which fill the knowledge gap that our planned work is intended to do) or refinement (e.g. improved methods of fish capture or handling, or advances in tagging methodology). Any advances in these areas during the lifetime of the Project licence will be discussed with the AWERB team and incorporated into the project.

16. Understanding immune responses to complex vaccines

Project duration

4 years 0 months

Project purpose

- Basic research

Key words

vaccine, gonorrhoea, outer membrane vesicle, antigen, parasite

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 study aims to understand immune responses to complex vaccines, which consist of many different components. The central scientific question is how these combine to induce an immune response which protects against disease in a vaccinated individual.

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?

Bacteria and parasites are responsible for many different infections which afflict both humans and animals. Of these, there are still many infectious diseases where there is no licenced vaccine available, or any such vaccine is of limited efficacy. Some vaccines contain just one component, such as a single protein. Others contain multiple components because they have been derived from, for example, a living bacterium. The general aim of

the project is to understand how different components within these types of complex vaccines contribute to an immune response. In principle, if we can improve our understanding of these responses, we can use that knowledge to design more effective vaccines.

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

Outputs from the project will be in the form of publications and presentations at conferences.

Specifically, our academic output will examine how vaccines which are made up of multiple molecular components are able to elicit an immune response. An example would be a bacterial extract which is made up of many different proteins and other molecules. We propose to investigate how these different components combine to induce antibodies against many different proteins, and the relationships between them. As another form of output, the data will be shared with the scientific community and offers the potential for comparison with similar data collected from human clinical trials.

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

The primary beneficiaries will be researchers working in the development of vaccines against infections caused by bacteria and parasites. There will also be translational benefit for the pharmaceutical industry- basic research on the immunological mechanisms underpinning vaccines is useful in helping to guide commercial vaccine development which might contribute to combating antibiotic resistance, for example.

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

Our primary output will be publication in learned academic journals. We will combine this with presentation at scientific meetings and conferences. We will make our data available to other researchers on publication- this will be either through dedicated online databases, or as supplementary material included as part of the research paper. We publish papers on an Open Access basis- they are accessible to everyone, without the need to go through a paywall. The same is the case for the data repositories- the data will be freely available to download.

Species and numbers of animals expected to be used

- Mice: 330

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 species proposed for these *in vivo* studies of immunogenicity is the mouse. Mice are the lowest sensate mammal with a highly evolved immune system that closely resembles our own and are a wellestablished model for studying vaccine immunogenicity. Because their immune responses are well characterised, we are able to make use of technologies

which have been developed by others. A good example is the use of transgenic mice- animals where the genome has been engineered in a specific way to provide an immunological 'marker' which we can use to study how this class of vaccines work. We will use younger (adolescent) mice because they are, from an immunological point of view, naïve- they therefore give us little background in our assays, which would arise from immunological memory.

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

Typically, an animal will be subject to administration of vaccine components by injection. The animal will experience brief and minor discomfort arising from each injection; such administrations will be at least 4 days apart, generally more than a week. No more than 3 doses of any vaccine will be given to any mouse. In the final procedure, the animals will be anaesthetised to collect blood.

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

From our experience, we expect adverse effects to inoculation of vaccine components to be minimal. We pre-screen our vaccine formulations to ensure that they are unlikely to provoke an inflammatory response. In addition, we carry out pilot studies with any new vaccine component or composition, to minimise adverse effects. Some animals may experience localised swelling or reaction at the point of injection, which generally fades after 2-3 days (which we would classify as a moderate effect). Even in these cases, we would not expect any discernible effect on animal 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)?

mild (most); moderate (some)

75% mild, 25% 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?

Prediction of immune responses to vaccination in animals is extremely difficult. One reason is that an immune response is dependent on the coordination of responses from multiple different types of immune cells located in different tissues in the body. Immunogenicity- the ability to provoke an immune response- is dependent on multiple

factors, including the nature of the molecular species in the vaccine. At present, our understanding of how these various factors operate to produce an immune response is poor. Sadly, we are unable to reproduce these effects reliably using in vitro or in silico (computational) models. One of the ultimate aims of this research programme is to improve this predictability through better understanding and quantitative measurement, which will ultimately help to replace the use of animals. In parallel, we are working with data from human subjects to develop models which would reduce the need for animal experimentation.

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

We have incorporated non-animal alternatives into the experimental programme to ensure that adverse impact of any vaccine tested on the animals used is minimised. This includes the use of cells cultured in the laboratory, which can be used for some measurements. As part of the study, computational methods are also used to select the most promising protein antigens and eliminate any which are predicted to cause adverse reactions. Our vaccine samples are also tested for any contamination which might cause an adverse inflammatory response.

Why were they not suitable?

There is no single non-animal alternative which can accurately reproduce the complex immune responses to a vaccine in an animal. This is because the complexity of the responses requires the interaction of too many cells and components in a way which, currently, cannot be reproduced outside a live animal. The alternatives are valuable in refining the vaccine compositions, but they only test very limited aspects of the immunogenic response induced by each sample. An immunogenic response is dependent on many different factors- the nature of the protein(s) used, the dose, the manner and timing of administration and others. It is currently not possible to predict the amplitude and type of immunogenic response which a vaccine will induce but it is vital to have this information before moving to human 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?

The numbers estimated are based on refinements that we have introduced under the previous licence, which allowed us to reduce the number of animals needed through careful controlling of experimental variables and appropriate experimental design. We typically now use mice in groups of 5, which is sufficient for us to be able to obtain statistically valid measurements of the stimulation of antibody responses when comparing vaccinated animals with controls, who receive an inoculation without the vaccine components. Each experiment can have 5 or 6 groups, which translates into 25 or 30 mice in each experiment, based on current numbers. The total number of experiments has been calculated to allow us to explore the variety of samples required and conduct sufficient repeats to ensure that our findings are robust and reproducible.

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

We have made use of previous experimental data to refine experimental design in an iterative manner, reducing animal number to a minimum needed to obtain a clear answer to the experimental question posed. Our experimental design compares responses between groups of animals who are administered the same vaccine and makes pairwise comparisons to derive statistically valid conclusions about the effects of particular parameters (eg the effect of certain antigens, dosage level, inclusion of adjuvant). Using these estimates, we have devised an experimental strategy which allows us to derive the maximum amount of necessary information for the minimum number of animals tested. This generally takes the form of an investigation into the strength and direction of immune response to a particular vaccine.

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 used computational methods and studies on isolated cells to screen our vaccines before administering them to animals. Computational methods allow us to identify which parts of each specific protein antigen are most likely to elicit an immunogenic response; such methods are not infallible, but they provide a useful basis from which to refine our vaccine composition. In addition, we make optimal use of the tissue from each animal, making multiple measurements from each individual. For example, we can break down the antibody response to a complex vaccine into its components, and thus understand in more detail how antibody response patterns may align with protection elicited by a vaccine. We incorporate animals used from pilot studies into experimental groups, thus reducing the total number of animals used. Finally, we will use an iterative approach, through cycles of experiments, to identify the most important parameters and optimise them as efficiently 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.

We have selected the mouse as the model organism for these studies, for the following reasons:

The mouse is the lowest sensate organism that can practicably be used for vaccination studies which will be translatable to humans.

The mouse immune system is well studied: immune assays (such as cell fractionation) are therefore well established, and suitable reagents for them are readily obtainable.

Mouse genetics is well understood: we can make use of transgenic animals which have been made by other investigators.

Our experimental plan reduces vaccination to the simplest and least obtrusive method possible- up to three inoculations per animal, delivered over several weeks. Animals will be handled in such a manner as to minimise stress during inoculation. As detailed above, we will make extensive efforts to ensure that the administered vaccine material is not harmful or likely to trigger adverse responses. Safety and the avoidance of unwanted side-effects is an extremely important aspect of vaccine design. All samples will be tested on a smaller group of animals first, to verify no unanticipated adverse reactions. All animals will be monitored in the 48-hour period after inoculation for weight loss and any signs of distress or discomfort.

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

We require animals with an immune system which is as closely similar to humans as possible. Less sentient species have immune systems which are too different from humans, so the results provide a poorer indication of the likely responses in a clinical trial. Mice are a well-established model for vaccination studies: our findings will therefore be easy to relate to those by other investigators who have carried out related, but different, experiments. Non-mammalian animals differ too much in their immune function from humans to be useful for vaccine studies. The use of embryos or very young animals is also not feasible because immune systems need to develop to a mature stage before they can be used as suitable models for the human immune response. Terminally anaesthetised animals cannot be used because it takes weeks for immune responses to develop and it is not feasible to keep animals under anaesthetic for that long.

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

Pilot studies will be carried out whenever a novel vaccine composition is introduced. All animals will be monitored after vaccination for reaction at the site of injection 6 and 24 hours after inoculation. In addition, animals will also be monitored in the 48-hour period after inoculation for signs of distress and altered behaviour (eg change in weight, hunched posture, reduced activity levels, altered social interaction). Observations of adverse reactions will be used to inform the design of future vaccine formulations- for example, we may reduce the level or number of doses where a particular vaccine component is the cause of the reaction.

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

We will make use of:

The PREPARE guidelines (https://norecopa.no/prepare) provide resources on study preparation, dialogue, education, communication and quality control of sample.

The ARRIVE guidelines (https://arriveguidelines.org/about) which covers similar topics to PREPARE, but extended to manuscript preparation and review. Many scientific journals now require completion of an ARRIVE checklist before publication.

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

We will use the newsletter and website of the NC3Rs, which is a valuable hub for dissemination of good practice in animal experimentation, with particular attention to innovations in the study and development of vaccines. We will also incorporate good practice from other specialists in the area: a good example is working with national bodies involved in replacing, reducing and refining animal use for vaccine testing. Staff engaged in the project will, in addition to mandatory training, be encouraged to engage with relevant webinars from NC3Rs and elsewhere which provide up-to-date advice and information on best practice.

17. Immune phenotype and dynamics in ocular autoimmune disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Autoimmunity, T cell, Macrophage, Dendritic cell, Microglia

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?

To identify and evaluate mechanisms that regulate autoimmune diseases, especially those effecting the eye, and to test new treatments that may improve disease outcomes in humans and 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?

Immune-mediated inflammatory diseases of the eye (known as uveitis or iritis) are a growing medical problem. In the USA, EU and UK, autoimmune uveitis is the 5th leading cause of preventable blindness. The disease is commoner in young adults, so has a relatively greater impact on patients than blinding diseases of old age. Steroids are the commonest first line treatment however, their use is associated with local and systemic side effects which, all too often, lead to poor treatment compliance resulting in a

suboptimal outcome for the patient. Consequently, there is a need to develop target specific treatments that don't rely on the use of steroids.

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

The outlined work will generate new information on the mechanisms (i.e. molecules and signalling pathways) involved in initiating and sustaining uveitis. In so doing, the work aims to advance understanding of the fundamental causes of this disease. In addition, the work will generate data on the effect of interventions aimed at modifying the course of disease with the aim of identifying improved treatment options for patients and animals. The data generated will be disseminated in presentations at scientific conferences and publications in peer reviewed scientific journals.

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

The work is expected to be of benefit to scientists, both nationally and internationally, working in academia and industry who are researching to find more effective treatments for autoimmune diseases like uveitis. Short term these benefits will be communicated through local meetings and medium term through publication of relevant results in journals and at international meetings. In the long term, it is to be expected that the data generated will contribute to the identification of more effective treatments for autoimmune disease and thereby benefit patients, medical professionals, health care providers and society. For example, as described below, previous research in animals by our group studying the role of Tumour Necrosis Factor in uveitis in mice contributed to the eventual approval by NICE of biological therapies targeting TNF in human ocular disease. These are now in clinical use treating uveitis in children and adults.

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

The scientists involved in the work conducted under this licence will actively participate in local research seminars and meetings where the work will be presented and discussed with collaborators and peers. The findings of the work will be presented to the wider scientific community via presentations at scientific conferences and publications in peer reviewed scientific journals, including presenting negative data where that will help other investigators identify unhelpful or unproductive lines of enquiry. Some of the studies will generate large data sets describing gene and protein expression in autoimmune disease and this data will be deposited in appropriate repositories where it will be available worldwide to other investigators, reducing the need for others to repeat similar disease model based experiments.

My institute proactively creates public engagement events with the public and patient that ensures our research addresses patients' needs. Our research will contribute to raising awareness of uveitis at these events, including explaining to a lay audience the importance of well-conducted model-based studies in the identification and development of new therapeutic interventions.

Species and numbers of animals expected to be used

- Mice: 4500

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.

Autoimmunity arises because the response of the immune system to threats such as infectious disease and cancerous cells is founded on a complex network of interactions that cannot be fully determined. Sometimes, the cells of the immune system attack healthy tissue and cause disease.

Many different components of the immune system are involved in the development of autoimmunity at the same time, including cells of the adaptive immune system (T cells and B cells), cells of the innate immune system (macrophages and neutrophils) and specialised cells such as dendritic cells and microglia. Furthermore, these components exist in a three-dimensional tissue matrix whose spatial properties play an important part in the immune response and the response occurs across anatomically distinct organs. While it is possible to use in vitro and modelling systems to interrogate individual molecular interactions, it is infeasible to model the totality of these process in vitro.

For the outlined studies, mice will be used to model uveitis as they have an immune system that is sufficiently similar to humans to enable valid generalisations to be made from the experimental outcomes, as demonstrated by their use in the development of many drugs used currently for the clinical treatment of humans. Furthermore, the models of uveitis we use are well established for mice. Because they involve the back of the eye, they effect the retina and, like the equivalent human diseases, may disrupt vision, but are not painful. In rodents this has minimal impact on wellbeing and does not result in behavioural changes because rodents are not heavily dependent upon vision for their daily activities due to their nocturnal nature. The long-term worldwide investment in understanding mouse immunology has also produced many refined genetic models that facilitate the experimental interrogation of immune responses. Important refinements include the ability to label cells and molecules of interest in vivo with fluorescent proteins and methods to inactivate individual genes in specific cell types during an autoimmune disease process. The outlined experiments will use juvenile and adult mice as it is essential that the immune system is fully developed and no longer influenced by maternal factors.

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

The majority of animals used for the outlined work will be bred in house with genetic alterations that do not cause any harm but which either enable specific cell types to be identified or which down regulate the animal’s immune response. Most animals will undergo a procedure to induce ocular autoimmune disease either by subcutaneous injection and/or intraperitoneal injection. Thereafter, the progression of the disease will be monitored clinically by the periodic collection of small blood samples and by imaging of the eye through a specialised microscope, while the animal is maintained under general anaesthesia. In some cases, animals may be treated with agents aimed at restoring normal function given either directly into the eye, following the induction and maintenance of anaesthesia, or by injection or oral administration. In some instances, the animal may be given the agent on repeat occasions or via a slow release device implanted surgically, following the induction and maintenance of anaesthesia. At the end of the study the animals will be humanely killed to enable tissues to be collected for analysis.

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

The genetic alterations used to label specific cell types have no impact on the animal's wellbeing, neither does down regulation of their immune response under the laboratory conditions that the animals are kept at. The injection used to induce the disease model causes mild transient pain but all animals are expected to resume normal behaviour within a few minutes. The disease state induced compromises the animal's vision intraocular injections that cause local bleeding, while not painful, may also compromise the animal's vision, but this does not have a significant impact on their wellbeing as mice are nocturnal and do not rely heavily on vision to move about their environment and find food and water. Furthermorewater. Furthermore, the disease state is not thought to be painful (based on previous studies and the knowledge that the associated condition in humans is not usually painful). The blood sampling used to monitor the progression of the disease causes mild transient pain, but all animals are expected to resume normal behaviour within a few minutes. Examination of the eye is performed under general anaesthesia, as is any treatment given directly to the eye, and all animals are expected to recover uneventfully. Animals given treatment orally or by injection will experience mild transient discomfort or pain but are expected to resume normal behaviour within a few minutes. Those animals undergoing surgery to implant a slow release delivery device are expected to experience some pain upon recovery from anaesthesia, which will be mitigated by giving them pain killers until they are showing no detectable signs of pain.

Animals may be injected with adjuvants that have the potential to cause harm (e.g. skin ulceration). This is minimised by staff training, the use of the most refined appropriate adjuvant, by subcutaneous injection in small volumes and in areas of loose skin by giving pain killers if they are showing detectable signs of pain. Animals may be irradiated to allow the transplantation of bone-marrow which may lead to weight-loss and susceptibility to infection, which is managed by monitoring and the provision of sterilized food under a defined standard operating protocol.

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?

Autoimmune disease is an emergent condition of the whole intact immune system that cannot be modelled in simpler systems.

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

Cell culture system and organoid studies are useful for investigating specific molecular pathways, but do not give comprehensive information on changes in the whole disease process.

Why were they not suitable?

Autoimmune disease is an emergent property of a complex interplay between many different cell types. As of yet, there are no non-animal models that successfully replicate the processes and, because of the lack of current understanding of the interactions involved, there is little likelihood of any being developed in the foreseeable future. We have previously published work using in silico models of immune responses, but these are not applicable to discovery studies for novel pathways.

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 largest number of mouse use relates to underlying breeding and screening of genetically animals modified animals, which we estimate at 200-300 per colony per year. Based on the reasonable expectation of the laboratory's capacity over the next 5 years, additional experimental animals will be purchased, and the estimated number is based on data from previous licence and expected income from grants over the period of this licence by we arrived at this final estimate.

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

Online tools such as the NC3R's experimental design assistant will be used to estimate the optimum animals numbers to use to detect significant effects in experiments using novel treatments. We have used clinical data from completed experiments to develop new approaches to quantification of disease using machine learning that reduces the number of animals that are needed for studies.

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 continue to be used to estimate effect sizes of novel interventions in the autoimmune process, where the magnitude of the expected effect needs to be estimated for power calculations (discussed below). Serial clinical monitoring increases the power of experiments to detect effects compared with single end-point experiments. To minimise animal usage, sharing tissue from other experiments will be used wherever feasible.

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.

Experimental autoimmune uveitis (EAU) will be used as the primary model for the outlined studies. This model leads to non-painful changes to the eye of the mice that closely mimic uveitis in humans and can be monitored clinically using sophisticated imaging methods. The condition produces little or no distress in mice and the impairment in their vision does not result in any behavioural changes or compromise their ability to navigate their cage and find food and water.

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

In order for the finding to be clinically relevant, it is essential to use an animal with an immune system that closely replicates that of humans. Consequently, mice will be used as they are the least sentient of laboratory species suitable for these studies and their immune system has been the subject of extensive research.

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

The animal model and procedures used for these studies have been extensively refined during previous work undertaken by my laboratory and collaborating colleagues. Whilst the disease condition induced does impair the animal’s sight, this has minimal impact on their wellbeing as mice are nocturnal and do not rely heavily on sight to navigate, find food and water or interact with their cage mates. The actual experimental procedures used during the study have all been refined under previous work and either cause no more than mild transient pain or are conducted under general anaesthesia. For example we have developed effective methods of isoflurane anaesthesia that support ocular imaging and rapid recovery. All surgical procedures will be conducted under general anaesthesia and the animals will be given pain killing drugs upon recovery, which will be maintained until the animals are showing no detectable signs of pain.

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

All procedures will be conducted in accordance with LASA and NC3r guidelines.

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

My institute and its AWERB committee, place a strong emphasis on promoting the uptake of 3Rs developments through seminars, open forum discussions and local and regional meetings. In addition, our Named Information Officer champions the 3Rs and ensure the

rapid dissemination of relevant initiatives to researchers. I also regularly attend and present at scientific conference with a 3Rs emphasis.

18. Tracking local pathology and systemic inflammation in arthritis

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

Inflammatory arthritis, Joint pathology, Systemic inflammation, Therapy, Cytokines

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?

Our research aims to define the cellular and molecular mechanisms driving joint pathology and systemic disease in inflammatory arthritis. Aligning investigations in mice with studies of human disease, we aim to improve the diagnosis, stratification and treatment of rheumatoid arthritis patients through an increased understanding of disease heterogeneity and multimorbidity.

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?

Inflammation is required for the healing process and is the body's response to infection, trauma or injury. In immune-mediated diseases, the control of inflammation is disrupted

and instead drives localised disease processes and more widespread complications such as cardiovascular disease, anaemia, fatigue and depression. Patients with immune-mediated diseases, such as rheumatoid arthritis, show features of disease that extend beyond joint inflammation (termed synovitis) and joint damage. Despite advances in the treatment of rheumatoid arthritis and other forms of inflammatory arthritis, not all patients respond to these drugs, and it is accepted that multiple, often independent, mechanisms contribute to disease progression. Protocols described in this licence will define the mechanisms underlying these clinical differences and are designed to improve patient diagnosis and treatment.

Considering local joint pathology- Early intervention remains the most effective therapy for rheumatoid arthritis. Current therapies are based on reducing inflammation and include inhibitors of proteins (termed cytokines) that instruct the course of inflammation. These include medicines such as blocking antibody therapies and oral drug inhibitors that have revolutionised the clinical management of patients with immune-mediated inflammatory diseases. Despite these advances, patients frequently fail to respond to a specific class of drug with clinical criteria currently unable to predict a patient's response to a targeted medicine. Advances in biopsy sampling of joints from the hands and wrists of patients with early arthritis show that the histological features of the inflamed joint are highly heterogeneous. These differences in disease presentation affect the severity and rate of disease progression, and response to targeted therapies. The cellular and molecular mechanisms accounting for these differences in disease are currently unknown.

Considering the broader clinical features of rheumatoid arthritis- Patients with immune-mediated inflammatory diseases commonly suffer complex multimorbidity that negatively impacts their clinical management. These include elevated risk of cardiovascular disease, anaemia, various metabolic disorders, diseases of the eye and symptoms of depression, fatigue, anxiety and insomnia. The mechanisms explaining the incidence of these conditions in patients with diseases like rheumatoid arthritis are poorly understood with these symptoms having a direct bearing on patient outcomes and quality of life. Extending the utility of the animal models described in this licence, approaches will consider the wider impact of localised inflammation (e.g., through antibody priming, induction of joint synovitis) on multimorbidity by introducing alternate methods to examine the link between local joint pathology and changes in systemic inflammation.

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

This project will provide new information on the immunological processes governing disease heterogeneity in patients with rheumatoid arthritis. The approaches will consider the functional properties of the immune cells involved in joint pathology, their interactions with the structural cells within the inflamed joint, and the wider impact of arthritis on associated co-morbidities (e.g., psychiatric well-being, cardiovascular risk, anaemia). Information obtained through these protocols will provide insights into the pathways driving inflammation in arthritis and are designed to improve understanding of patient outcomes in response to biological or targeted medicines commonly used to manage the symptoms of rheumatoid arthritis and co-morbidities. Research findings will be disseminated to the scientific community through presentations at national and international conferences and in peerreviewed publications in scientific journals.

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

The science is based on a committed basic and applied research programme with potential for future exploitation and clinical translation. Applying fundamental mechanistic

studies and validation approaches in studies of immune-mediated inflammatory diseases can deliver on several levels.

Research community– The immediate academic beneficiaries will be researchers in the field and aligned disciplines (e.g., immunologists, cell biologists and geneticists). The research aims to advance our understanding of chronic disease progression, the dysregulation of inflammation, and the processes steering inflammation-induced tissue damage. While primarily of interest to those working on immune-mediated inflammatory diseases, the questions being addressed have broad appeal to other distinct medical disciplines and those interested in disease mechanisms. For example, conditions including hepatic liver disease and cancer, where similar mechanisms of disease pathology appear to operate.

Patients– Patients with immune-mediated inflammatory diseases show varying efficacies to biological drugs that inhibit cytokine activities. Discussions with rheumatoid arthritis patients emphasise the challenges associated with the management of their condition. These include the need to take ownership of the disease, by not allowing their disease to rule their lives. Here, conversations emphasise the importance of an established and effective therapy that enhances their quality of life and provides confidence that their condition is appropriately managed and controlled. The research aims to understand the underlying mechanisms of joint inflammation responsible for disease heterogeneity in rheumatoid arthritis and the potential lack of efficacy displayed by some patients in response to certain therapies. Thus, we aim to improve the diagnosis and longer-term clinical management of patients with rheumatoid arthritis. Patients and awareness groups embedded in our research programmes have informed the development of the research. Their participation is instrumental in furthering the translation of our research, promoting awareness, partnerships with patients, opportunities to participate in the research, and patient forums to discuss research findings and prioritise future studies.

Doctors and healthcare professionals– A long-term objective is to identify new hypotheses that support the clinical management of patients with varying forms of synovial inflammation. To this end, we work with recognised specialists in experimental rheumatology and stratified medicine to align fundamental discovery science in mouse models of arthritis with data from clinical studies and experimental medicine protocols.

Time frame: Our laboratory routinely uses mouse and human data to substantiate findings and new hypotheses. We anticipate that data generated under this licence will increase our understanding of arthritis progression and improve decisions on the best course of therapy. However, additional time beyond the licence would be required to realise the clinical utility of any innovations arising from the research.

The pharmaceutical sector– The protocols offer the potential to build meaningful collaborations with industrial partners. These include opportunities to test the efficacy of novel drugs and therapies developed within the pharmaceutical sector and reciprocal working relationships, where novel intellectual property generated as part of ongoing research may be commercialised for clinical benefit.

Time frame: Our prior interactions with the pharmaceutical sector reveal two levels of engagement. (1) Investigator-led or contract research where the pharmaceutical sector collaborates to test the efficacy of biological or targeted medicines in our model systems. These interactions typically involve the development of modalities that target our pathways of interest or help address our experimental objectives. These *ad hoc* interactions may arise within the licence period (2) Support to advance innovations identified by the

laboratory during our research. These take longer to develop and our experience with olamkicept suggests that this might exceed 10 years.

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

Fundamental discovery science will open new avenues for clinical innovation and improved patient outcomes. Results will be disseminated through research publications, presentations at scientifically relevant conferences and workshops, and educational events for healthcare professionals and the commercial sector.

We will engage researchers and clinician scientists involved in clinical trials of pathological assessments and ultimately practising clinicians. While primarily of interest to the rheumatology community, the questions being addressed have broad appeal to other medical disciplines. These include clinical specialities treating inflammatory or immune-mediated conditions and cancers where cytokines direct differences in the underpinning pathology. Last, we will seek to collaborate with other clinical specialities through participation in leading scientific conferences and workshops. The Applicants are regularly invited to lecture at meetings where arthritis, immunology, cytokines, pathology, and inheritable genetic disorders are major themes.

The research obtained through the outlined procedures will attract interest from those involved in precision medicine and the development of biological drugs, small molecule interventions, and diagnostic innovations. Here, the approaches used, and the application of our findings may help to differentiate between the mode-of-action of emerging biological therapies. We actively engage with the pharmaceutical sector and sit on advisory boards relevant to the development and clinical translation of these drugs– e.g., Roche, Chugai, Genentech, GSK, Sanofi, NovImmune SA, Ferring, and Janssen Pharmaceuticals.

The study design will generate whole-genome datasets of transcriptomic and epigenetic mechanisms within the inflamed joint and associated lymph node tissues. Published discoveries will be deposited in open-access data repositories (e.g., Gene Expression Omnibus) that are freely available to researchers studying similar questions. There are currently no freely available datasets relating to the involvement of transcription factor signalling in the processes described in this licence application. Analysis of these datasets will likely involve generating new computational strategies (e.g., R-scripted methods). These will be made freely available on request. Understanding the link between inflammation and disease heterogeneity is becoming a topic of increasing interest requiring alternate methods and approaches that bridge, immunology, genetics, genomics and pathology. To exploit these potentials, we will collaborate with data scientists, bioinformaticians and mathematicians to improve the analysis of generated datasets.

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.

Pre-clinical models of inflammatory arthritis in adult mice are well-established, and reproducible and have contributed to the pharmaceutical development of biological and

targeted medicines commonly used to treat patients with rheumatoid arthritis and other forms of arthritis. Mice share a comparable neurophysiological sensitivity to humans and the historical research into immunological processes has provided an extensive panel of reagents to study immune responses. This includes the capacity to use genetically altered (GA) mouse strains (including gene deletions, transgenic over-expression, and knock-in mutations to alter molecular and cellular functions) that resemble human immune activities. Importantly, our prior understanding of disease processes and immune activities in mice allows animal usage to be decreased by focusing on the aspects of disease that increase our chance of obtaining interpretable and meaningful data in our experiments.

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

Introductory comments on animal maintenance and well-being during the outlined procedures:

Animals will be housed in environments with appropriate quantities of food, water and bedding. The procedures involved in this study are well established and can be conducted in a manner that offers maximum information but minimal distress to the animal. This experience has also allowed us to define early end-points for experimental protocols that involve the induction of inflammation and arthritis and help reduce animal suffering to a minimum. Where appropriate, analgesics are routinely administered early to avoid the symptomatic pain associated with arthritis development. All animals are given environmental enrichment (chew toys, tunnels) to express normal behaviours. Where possible, animals are group housed, with caging providing bedding to shred and food treats such as sunflower seeds. To reduce unnecessary stress and anxiety, all mice undergo habituation before experimental procedures. To alleviate discomfort and joint pain for mice with arthritis, we routinely use bedding materials less prone to tangling. For mice with arthritis, we seek to minimise unnecessary handling and reduce discomfort by conducting handling and restraint on soft VetBeds.

Information on outlined procedures:

Mice will be used to characterise the immunological processes steering the progression of joint disease and associated co-morbidities in inflammatory arthritis. Before experimentation, mice will be acclimatised for one week in the facility. This acclimatisation period is particularly relevant to mice purchased from accredited breeding facilities. Mice will be treated with substances promoting immunological responses resembling inflammatory or autoimmune reactions in human disease. These will mostly involve administration by injection (under the skin, into the knee joint or abdominal space) while under general anaesthesia. Mice will be monitored for clinical signs of inflammation or arthritis. In some experiments, we will test the therapeutic properties of immune-modulating agents delivered by injection or other routes of administration. Small volumes of blood (from superficial vessels), urine, saliva, or pieces of skin tissues and hair, may be taken. Analysis of these samples will track immune or metabolic responses to disease or genetic modifications. Most experiments will last 1-2 months before mice are killed using a Schedule 1 method or by exsanguination or perfusion fixation performed under general anaesthesia and tissues recovered for analysis. To track the impact of arthritis onset on comorbidities, mice may be placed in environments that allow measures of metabolic processes (e.g., metabolic cages that capture urine samples) or protocols that record changes in animal behaviours such as mobility or mental well-being. These protocols will be conducted following an appropriate habituation.

Genetically altered (GA) mice will be bred to evaluate the involvement of specific immune pathways in joint inflammation and associated co-morbidity. These mouse strains will

include those with gene deletions, transgenic over-expression, and knock-in mutations to alter molecular and cellular functions. In addition to the experimental procedures outlined within the licence, these mice will also be used to support *in vitro* and *ex vivo* studies into the cellular mechanisms determining their functions.

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

Mice will be administered agents that promote inflammation or arthritis. No lasting discomfort or irritation is anticipated in mice injected with an immune activator. However, skin irritation or damage may arise in mice receiving multiple treatments. These adverse events rarely persist beyond 24 hours, and animals typically display no signs of ill health. We are, however, mindful that certain immunedeficient genetically altered (GA) strains may be more susceptible to these reactions, and appropriate steps are taken to monitor and reduce the exacerbation of skin irritation. A specific risk assessment based on published literature or known responses to immunological challenges should be undertaken to identify potential deleterious phenotypes (e.g., alternations in bone turnover or homeostasis affecting mobility) and adverse reactions and the likelihood of mice extending the severity limit of the experimental protocol. These traits should be considered for all GA mouse strains entering protocols for the first time.

For mice primed to develop arthritis, joint pathology leads to a loss of mobility, due to an initial inflammatory swelling of the limb. This swelling typically resolves within 48-72 hours following disease induction. During this initial period, treated mice show slightly reduced exploratory behaviours. Some mice will be used in a model of chronic inflammatory arthritis, where they typically experience joint swelling lasting 8-10 days before they are killed for tissue analysis. Where possible, suffering will be minimised by analgesia and regular monitoring of the mice for any clinical signs of suffering (e.g., behaviour, piloerection, weight loss). Rarely, mice may experience an adverse response to reagents that promote arthritis onset, such as skin irritation at the injection site. In all cases, mice will be carefully monitored for unexpected adverse events. If signs persist and are not prevented by mild veterinary intervention, they 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)?

54% Mild and 46% 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?

At present, there are no alternatives to the *in vivo* models described. Clues as to how arthritis develops reside in the pre-clinical stage (where symptoms may not be apparent to the patient) and where the initial triggering and induction events that lead to arthritis development take place. Unfortunately, this phase of human arthritis is not readily accessible for investigation and therefore remains speculative. While *ex vivo* studies of tissue extracts or *in vitro* cell culture protocols support investigations of immune-stromal cell interactions, the protocols outlined provide additional opportunities to explore the systemic features of arthritic inflammation and the impact these processes have on the local tissue within the inflamed joint. In this regard, the described protocols provide the possibility of tracking longitudinal and temporal changes in immune regulation throughout the disease course.

Rodents provide excellent species for experimental models of arthritis. They are most likely to produce satisfactory results because of their similarities to the human response. Rodents provide the lowest species with comparable neurophysiological sensitivity to humans. Rodent models are most commonly used, due to cost and homogeneity of the genetic background. In mice, there is also the capacity to use GA strains, furthermore, immune responses in mice closely parallel those in humans. Genetic mutations required for extensive and detailed analyses of immunological function are available and already characterised in mice.

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

We use a variety of in vitro model systems in our laboratory, such as cell lines (e.g., fibroblast-like synoviocytes, chondrocytes, leukocytes and osteoclasts), synovial tissue explants (human, bovine and murine) and clinical samples (blood, serum and synovial fluids) originating from patients with rheumatoid arthritis, osteoarthritis or volunteers unaffected by arthritis. For example, we routinely compare results from mice with arthritis to data from tissue biopsies obtained from patients during the early stages of rheumatoid arthritis or those monitored following treatment with biological medicines or targeted therapies.

Why were they not suitable?

While in vitro approaches allow the mechanistic study of individual cellular responses, they cannot recapitulate the complex network of cellular communication and migration that occurs in vivo during inflammation. The immune processes responsible for the development of arthritis likely occur before the presentation of clinical signs and symptoms of joint disease (i.e. when the disease may not be overtly apparent to the patient). These early warning signs will cascade the escalation of inflammatory processes leading to the onset of arthritis – affecting the severity, the rate of disease progression and the response to therapy. Unfortunately, this initial phase of arthritis is difficult to track in humans and remains subjective. At present there is no alternative to using the in vivo models described to achieve the experimental objectives outlined in this 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 success of our previously approved licence (PE8BCF782) has allowed us to generate archived datasets that can be used to support the aims and objectives of this current application. This includes banked tissues from the joints of mice with arthritis, next-generation sequencing data offering information on gene regulation following disease onset and the cell types involved, and histological evidence from drug inhibition of immune pathways that support the design of future experiments. These resources will reduce the anticipated number of experiments to be performed.

Group sizes for experiments are based on results from previous experiments (i.e., previously observed effect sizes) or a standardised effect size (e.g., Cohen's d of 1.5) for pilot studies where no previous data are available. Typically, our experiments require between 6-10 mice/group. These numbers are, however, dependent on the outcome measures being considered. It is estimated that 500 mice/year will be used in the experimental protocols of inflammation or arthritis. These numbers are required to meet the scientific objectives of currently funded projects and grant applications under review or in preparation (approximately 8-12 experiments/year; with 2 independent repeats).

The numbers used for breeding and maintaining genetically altered (GA) strains equate to 500 mice/year. These mice will also provide tissues for *in vitro* studies supporting the *in vivo* models.

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

Online experimental design tools (e.g., the NC3Rs experimental design assistant and its power calculator tool) adequately power experiments with sufficient animal numbers. Prioritising the need to identify biologically relevant changes, we also seek to avoid using too many mice. We also consult the statistical software tool G*Power (gpower.hhu.de) when power for more than 2 groups is needed, or where non-parametric analyses are used.

We routinely discuss our research with clinical colleagues to ensure we ask meaningful questions. Our previous research has yielded a large amount of data, archived samples for training purposes and informed decisions on the best study design (e.g., selecting specific time points within the model). However, our laboratory increasingly uses new methodologies to generate more information from fewer mice. These include next-generation sequencing techniques capturing whole genome changes in gene regulation as a response to disease onset. Moreover, we also use comparable datasets derived from human clinical studies to verify our mouse findings, and this approach often informs new questions that can be tested in our experimental model systems. This approach benefits from the fact that mice are well-defined immunologically, which decreases experimental variability and increases opportunities to generate meaningful data.

Historically, we have relied on histological approaches to generate an understanding of disease (e.g., qualitative measures of synovial infiltration, synovial exudate, synovial hyperplasia and joint damage). These measures require larger animal numbers to generate statistical significance. We are less reliant on these methods and typically use these approaches when testing mice where we have little understanding of how they behave in models of arthritis, or where we are testing pharmacological agents that may interfere with defined aspects of the pathology.

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

Experiments are designed to produce unambiguous results using the minimum number of mice. Consideration is given to mouse numbers/group and is based on our experience with models of arthritis, inflammation and animal behaviour (>15 years), and statistical power analysis (using

G*Power; gpower.hhu.de). Our research has examined joint pathology, systemic immune activation, animal gait alterations, and changes in behaviour. All inducible models show a strong penetrance (e.g., antigen-induced arthritis shows 98-100% penetrance in C57Bl/6 mice).

Where appropriate we will perform experiments using equal numbers of male and female mice. For certain experiments, we have already identified that male and female mice show no differences in immunological parameters (e.g., monitoring changes in inflammatory cytokines or immune cell recruitment) following immune challenge (e.g., activation of innate sensing pathways). For others, the penetrance and reproducibility of disease induction remain gender-specific (e.g., antigen-induced arthritis in male C57/Bl6 mice).

Appropriately powered experiments will be conducted once. If further statistical power is required, experiments may be repeated. All data will be reported as *per* ARRIVE guidelines. Sex-matched mice are used, which reduces variability. Experimental and littermate control mice in Aim-2a will be cohoused in cages, facilitating blinding. For treatment studies, the NC3Rs EDA ‘randomised allocation report’ will allocate mice to groups for blinding. Clinical assessments will be performed blindly, with joint histopathological scored by two independent observers blinded to the study groups.

A laboratory database records the breeding status of all mouse strains, the number of mice available for experimentation (including age, sex and genotype), and the assignment of individual mice to particular experiments. This approach was introduced following discussions with our Australian collaborators who adopted a similar system several years ago. The senior PIL holders in the laboratory manage this repository, and all staff members associated with the project have access to the database. The introduction of this database provides evidence of how we implement best practices from elsewhere to improve our management processes. In this regard, we have used this approach to help reduce the overall number of animals used. For example, maximise the experimental information that may be derived from each animal or group of animals (e.g., harvesting different organs for discrete sets of 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.

Inflammation is a complex process that requires communication between immune cells and resident tissue cells at the site of disease activity. Mice are an excellent model system for studies of inflammation and disease processes responsible for arthritis. These studies benefit from access to numerous genetically altered (GA) mouse strains, and the

availability of mouse reagents (e.g., antibodies, recombinant proteins) to track immune responses or to test the therapeutic properties of pathway inhibitors.

The procedures themselves will cause no more than mild transient pain or suffering. However, the majority of mice will go on to develop symptoms of inflammation or arthritis as a consequence of these procedures. These outcomes are classed as moderate severity. To minimise the impact of the outcomes, we prioritise arthritis models of lower severity and use studies of acute resolving inflammation to open new avenues of investigation. For example, >90% of mice that develop arthritis will be used in a model where mice develop self-remitting and resolving joint inflammation only in an injected knee (antigen-induced arthritis), without the more progressive and systemic (affecting multiple joints throughout the body) joint involvement seen in other models. The incidence of disease in antigen-induced arthritis shows 100% penetrance, with immediate and synchronous onset. This model develops over a relatively short time frame, which helps to minimise any lasting pain or discomfort.

The development of arthritis is monitored closely. Disease onset does not restrict the animals' ability to feed, drink and explore their environment. Some mice that develop arthritis (<10%) will be used in a more systemic poly-articular model of arthritis (i.e. affecting multiple joints). Here, the development of arthritis is closely monitored, does not impede the animals' ability to feed and drink, and mice are given analgesia at, or before, clinical onset.

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

Inflammation and disease progression are active processes that rely on live mice. The age of the mice is selected to ensure the 'immunological competence' of the mouse and the structural integrity of the synovial mouse joint is fully developed. On a practical note, the intra-articular administration of agents into the mouse joint means that the mouse has to be sufficiently large to ensure reliable and reproducible injection of the joint space.

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

We (in collaboration with others) have refined the described models through discussion with clinical colleagues and where possible we have adopted methods to focus on specific experimental questions. This has allowed us to refine the experimental protocols by:

Conducting a detailed temporal analysis of the inflammatory processes activated in response to arthritis induction- Tracking the parameters of disease, we have established how immune and stromal tissue cells behave in response to arthritis onset and have used this information to identified optimal times for their involvement within the model. This approach ensures that only high-value time points are chosen and mice are not subjected to needless procedures at non-relevant time points.

Reviewing imaging techniques that reduce unnecessary handling- Monitoring protocols often require excessive handling of the mice (e.g., measurement of joint swelling using callipers). This causes distress and anxiety to the mice (including those housed in the same cage), which may trigger immune activities in response to psychological stress. Adopting alternate imaging techniques means that certain measurements can be conducted only once at the end of the study once the animals have been killed. Working with imaging technologists we are exploring new ways to examine pathology – e.g., micro-CT to study joint and bone pathology, IVIS fluorescence to monitor the turnover in extracellular matrix.

Adopting steps that mitigate suffering and distress- Dermal injections are made into the rump and never at the base of the tail. Gaseous anaesthesia is used for all intradermal and intraarticular routes, to increase the accuracy of injection, decrease pain, and improve the ease of handling for experimenters. Volumes and doses will be limited to the minimum needed to induce effect, and multiple sites will be used if larger volumes are necessary. Agents such as CFA will be correctly formulated to minimise swelling and pain, further CFA will only be administered via a dermal delivery. Although we intend to use both AIA and poly-arthritic models (Collagen-induced arthritis), the majority of work conducted will pertain to monoarticular arthritis onset (AIA), as this model allows us to investigate factors that influence induction as well as resolution of arthritis, as well as causing much less suffering and distress to the animal, collagen-induced joint damage can is considered a more involved model and as such it is not possible to study resolution here. In addition for any collagen-induced arthritis work, opioid-based analgesics will always be used to reduce pain and distress.

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

We follow the advice and guidance outlined in published, peer-reviewed articles. These are specific to the use of arthritis models in mice. Examples include:

Hawkins, P. *et al.,* (2015). Applying refinement to the use of mice and rats in rheumatoid arthritis research. Inflammopharmacology 23: 131-150.

Vierboom, MPM. *et al.,* (2017). Pain Relief in Nonhuman Primate Models of Arthritis. Methods in Molecular Cell Biology (Inflammation, Methods and Protocols) 1559: 411–417.

Webb, D.R. (2018). Animal Models of Rheumatoid Arthritis. In: Ragab, G., Atkinson, T., Stoll, M. (eds) The Microbiome in Rheumatic Diseases and Infection. Springer, Cham. https://doi.org/10.1007/978-3319-79026-8_6

Oh, SS. & Narver, HL. (2024). Mouse and Rat Anesthesia and Analgesia. Current Protocols 4(2):e995 [doi:10.1002/cpz1.995]

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

Animal procedures conducted under previous or existing Home Office licences are regularly reviewed by the project licence holder, the staff performing the experimentation and animal technicians within our institutional unit. These include a review of current approaches and whether there are any new 3Rs opportunities. The section below provides some examples of changes we have made to improve the management of the research involving animals. We have also adopted improved practices through discussion with collaborators in other institutions and others working in areas outside our field of expertise (e.g., researchers of cancer).

Monthly updates described in the NC3Rs newsletter provide us with information on funding opportunities, events and publications, and institutional 3Rs symposia advance our understanding of novel 3Rs approaches, tools and technologies and experiences from elsewhere. This includes access to the konfer NC3Rs webpage (https://konfer.online/organisation/business/NC3Rs?id=13) showcasing relevant technology platforms developed by academia and smaller companies.

19. Investigating novel formulations & the delivery of vaccines and drugs

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

Novel, Formulations, Drugs, Vaccine, Delivery systems

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?

This research work aims to provide new formulations that may improve the efficacy of already available therapeutic agents or vaccines, facilitate the formulation of substances that might otherwise be inactive, and improve storage characteristics e.g., increased shelf life or heat stability, thereby avoiding the need for cold-chain vaccine handling (ensuring the vaccine is kept within a certain temperature range from the time they are manufactured until they are administered).

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?

Identification and characterisation of drug and vaccine formulations and delivery systems that improve stability or biological action could lead to significant benefits in terms of contribution to healthcare quality worldwide. In real terms, we expect that work undertaken in this project will help discover new areas – such as techniques and the use of novel agents – of drug and vaccine delivery systems that may provide the basis for further research, possible clinical trials and eventually more effective formulations for use in humans and animals. Therefore, the dissemination of our research from this project will hopefully result in the adoption of more effective formulation strategies within the pharmaceutical industry.

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

A greater understanding of the immune system, distribution of drugs and formulas of different compositions, gaining this information helps us to be able to adjust our compounds to improve the delivery system.

Identifying and characterising drug and vaccine formulations of interest and delivery systems that improve stability or biological action.

We would expect a similar output to the previous Licence. It is hoped that specific candidate drugs, vaccines, and formulations of interest that have been studied through the project may enter pre-clinical and clinical trials and be manufactured to benefit the human and animal population.

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

We are continually assessing new formulations and improving the delivery of existing drugs using our novel nanoparticle-based systems. We either manufacture these formulations or develop them in collaboration with our numerous partners.

Short-term benefits: Throughout the five years of this project, we anticipate generating new knowledge on the formulation and delivery of drugs/vaccines of interest. This includes understanding how specific nanoparticles enhance stability, targeting, or uptake. Such insights will be of immediate benefit to researchers in the field, providing a foundation for further advancements in drug delivery technologies.

Mid-term benefits: These efforts will feed into preclinical successes, including advancing promising candidates closer to clinical trials. For example, improved formulations will streamline pharmaceutical development pipelines, offering cost and time savings to the pharmaceutical industry. These developments will also help address critical bottlenecks in producing and delivering vaccines or therapeutics for diseases with high unmet medical needs.

Long-term benefits: The work has the potential to deliver widespread improvements in human and animal health. Depending on the candidates analysed, this research could contribute to developing safer and more effective medicines and vaccines. Examples include vaccines for tuberculosis (TB), respiratory syncytial virus (RSV), influenza, personalised cancer vaccines for humans, and vaccines for TB and rabies in animals. The outcomes of this project will significantly enhance healthcare quality on a global scale, benefiting both human and veterinary medicine.

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

Dissemination will be via delivery at conferences and peer-reviewed publications.

We have several collaborators around the UK and internationally, and of course, all funders will also disseminate the project's outputs.

Species and numbers of animals expected to be used

- Mice: 3960
- Rats: 90

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 immune systems and physiology of lower vertebrates are significantly different from humans, including restricted antibody diversity that may be partly due to the fact that some immunoglobulin segmental elements do not undergo genetic rearrangement (for example in fish) in the same way as that which is seen in humans (and that is also seen in mice).

For biodistribution studies and drug delivery we intend to use adult mice. The murine model is our model of choice as the mouse physiology, in terms of organs and systems, has many parallels to that of human systems. At present, it is impossible to recreate the biological environment in-vitro in terms of biodistribution throughout complex physiological systems following the administration of a therapeutic or prophylactic agent. For this and evaluation of biological effects in some systems (particularly the immune system mentioned here) animal testing is still necessary.

The ability to use rats is for certain studies where the sensitivity of the compound requires larger samples for testing at the end of the screening or for comparison studies where necessary. For example, in the testing of the drug delivery of drugs in nanomedicines (e.g., doxorubicin in liposomes), we need to measure plasma drug concentration more frequently, and rats are more suitable. Similarly, if a drug has a metabolism heavily influenced by cytochrome P450 enzymes, rats can make a better model than mice.

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

In protocol 1 animals will be administered substances (including radioisotopes) with or without anaesthesia, in the form of an injection (Subcutaneous (SC), Intramuscular (IM), Intraperitoneal (IP), Intradermal (ID) or Intravenous(IV)), inhalation, intranasally or by oral gavage, typically three times over the course of an experiment (but possibly up to seven times depending on the route), on a once every two-week schedule, this would be interspersed with taking blood samples up to 8 times over the course of the study (one before the first dose and then weekly). Animals will then be culled using either a non-schedule 1 method - cardiac puncture under anaesthesia (to obtain a good volume of blood for analysis), or by a schedule 1 method if blood sampling is not required.

Schedules will involve inoculations no more than twice in any one week and no more than six inoculations within the duration of the procedure for most routes but no more than three times in any one week and no more than seven inoculations within the duration of the procedure for the oral route.

In protocol 2 animals will be administered with the agent of interest and then imaged using noninvasive techniques such as the In Vivo Imaging System (IVIS) and/or Computed Tomography (CT) which requires them to be administered fluorescently labelled substances and/or contrast agents, this could be done with/without anaesthesia, by one or more of the following routes: intraperitoneal, intramuscular, subcutaneous, oral (gavage), intravenous. Optionally, a suitable dye (e.g. pontamine blue) is administered by the subcutaneous route. Alternatively, under general anaesthesia, substances may be administered via one of the following routes: instillation by the intranasal/inhaled route, intradermal injection or intratracheal gavage.

IVIS/CT non-invasive imaging involves anaesthetized animals being placed inside the imager for a limited time and then allowed to recover. Animals may occasionally be imaged for up to one hour, but typically for less than 15 minutes. Animals will be allowed to rest for a period of no less than 1 hour between imaging sessions. Any one animal may be imaged up to 15 times, with no more than 4 imaging sessions in one day (with the exception of pilot studies). A study may last 1 month, with imaging sessions spaced throughout. Based on our previous experience, imaging is normally performed at 10 min after injection, 6 h, 24 h, 48 h and 72 h and weekly there after.

In a minority of studies (e.g., when the agent does not luminesce) we might take a very limited number of blood samples (probably no more than two) to verify plasma levels.

Animals will then be culled using either cardiac puncture (to obtain a good volume of blood for the study) or by a schedule 1 method.

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

Protocol 1:

Drug Administration: No adverse effects are anticipated due to the drug delivery systems/drugs administered. However, when working for the first time with systems/drugs with unknown adverse effects, initial studies will be conducted using low doses and one mouse at a time to ensure there are no related toxicity issues.

Dosing itself can cause mild, momentary discomfort/pain. To reduce discomfort/pain as much as possible, the volumes administered will not exceed the recommended volumes stated in the establishment's dosing guidelines, and the mildest, most appropriate administration route will be chosen, taking into consideration the administered substance.

Blood Sampling: likely to result in mild, momentary discomfort/pain. To minimize this, the establishment’s good practice guidelines, based on recommended best practice, will be adhered to.

Radioisotopes: these will be administered at levels below therapeutic levels, and in our 15 years of experience appear to cause no adverse effects to the animals.

Mice injected with pontine blue get a bluish tinge in the skin, especially on the nose, ears, and paws. However, no adverse effects on health and behaviour have been observed when administering pontine blue.

Induction of anaesthesia results in mild, momentary stress and, when repeated, can induce a behavioural aversive reaction. These effects are minimised by good technique and by avoiding the use of anaesthesia whenever possible.

Protocol 2:

Drug administration: as described above for protocol 1.

Imaging: this is not an invasive procedure, but animals need to be still during the scanning session, and therefore, an anaesthetic is required. Induction of anaesthesia is stressful to the animal and, when repeated, it can induce a conditioned aversive behaviour. Therefore, the number and length of imaging sessions will be kept to the minimum possible to answer the specific experimental questions. Similarly, depending on the experiment, the longest possible interval between anaesthetics (to allow best recovery) will be used. Positive reinforcement techniques (e.g., offering treats) will also be used.

Prolonged and/or frequent anaesthetics may result in dehydration. If necessary, the animals will be given subcutaneous fluids in consultation with the NVS.

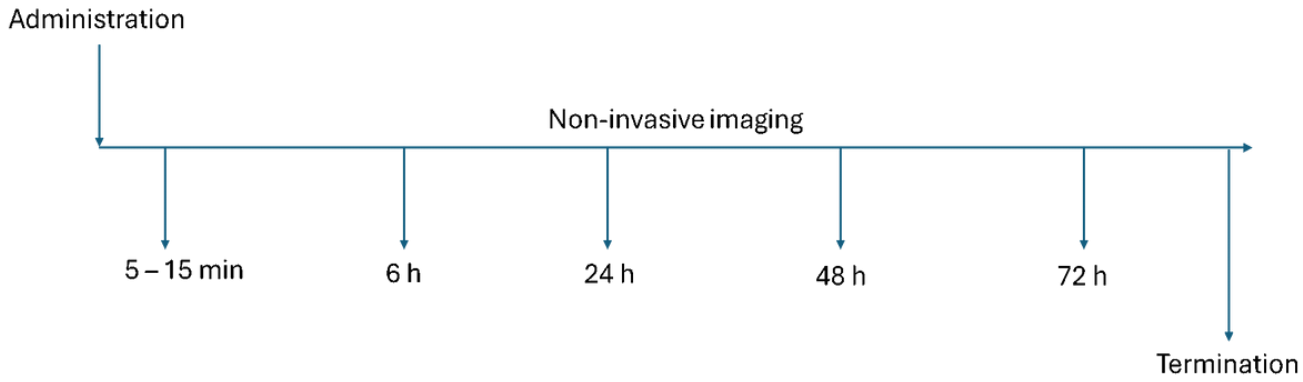


Fig. 1: Example of a study with one injection of fluorescently labelled substance followed by multiple imaging sessions. This is an example of a pilot study and fewer imaging sessions may be used in subsequent studies. Similarly, additional time points may be added to the study if mRNA is seen after 72 h .

Anaesthesia: the level of anaesthesia will be maintained at sufficient depth to achieve immobility (i.e., light general anaesthesia). Deaths resulting from general anaesthesia are most uncommon in our experience (<1%) and will be kept to a minimum by ensuring accurate dosing during gaseous anaesthesia and keeping the animals warm.

Blood Sampling (if required): likely to result in mild, momentary discomfort/pain. To minimize this, the establishment's good practice guidelines, based on recommended best practice, will be adhered to.

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- 60% mild and 40% moderate.

Rats - 100% 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?

After our extensive invitro testing of compounds, currently the only option available to us to track the progress of compounds within a complex system is to use live animals.

Unfortunately the immune systems and physiology of lower vertebrates are significantly different from humans, including restricted antibody diversity that may be partly because some immunoglobulin segmental elements do not undergo genetic rearrangement. However, humans and mice share many similarities in antibody diversity and immunogloblin elements. In addition, for biodistribution studies and drug delivery, the murine model is the model of choice as mouse physiology, in terms of organs and systems, has many parallels to that of human systems.

Rats will only be used where the mouse model is not adequate to supply the necessary quantities of blood required for analysis or where the murine model would not serve as an appropriate comparison point for a study.

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

In conjunction with our in vivo studies, we have used these to develop an in vitro testing model. We have published preliminary work on and are in the process of finalising research funded by Nc3Rs entitled ‘Towards an in vitro system of predictive biomarkers of in vivo liposome efficacy’. In these studies, we have quantified liposome uptake by phagocytes using the human continuous cell line THP1. Fluorescence-labelled liposomes were co-cultured with THP-1-derived macrophages at a final lipid concentration of 5 µg/mL. The proportion of macrophages associated with fluorescent liposomes, and the relative amount of fluorescence associated, was quantified using flow cytometry. Our results show the time-dependent uptake of liposomes after application to THP-derived macrophages at 37°C; with changes in formulation influencing cellular association in line with in vivo results we previously found.

We have also developed a rapid in vitro pre-screen for distinguishing effective liposomal adjuvants. We are continuing with these studies and using previously published in vivo data to back-correlate with the in vitro studies to further validate this in vitro tool and tools that can be applied to mRNA vaccines.

Why were they not suitable?

At present it is impossible to recreate the biological environment in terms of biodistribution throughout complex physiological systems following the administration of a therapeutic or prophylactic agent. For this and the evaluation of biological effects in some systems (such as the immune system mentioned here) animal testing is still necessary.

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 has been estimated based on our current funding and experience of screening formulations.

For rats, we typically will start this as a high-throughput in vitro model where we screen up to 20 formulations for the physical attributes . From this, at least 10 of these will progress into more detailed in vitro studies, and we then down-select a maximum of 2 formulations to test and include 1 control group. This gives us 15 to 18 animals normally split over two studies (to provide intra and interday variability). We would normally conduct up to 5 of these studies in rats throughout the project (covering protocols 1 and 2).

For mice, again we will start this as a high-throughput in vitro model where we screen up to 20 formulations for the physical attributes. From this, at least 10 of these will progress in vitro detailed studies, and we then down-select a maximum of 5 formulations to test and include 1 control group. This gives us 30 to 36 animals split normally over two studies (to provide intra and interday variability). We would normally conduct up to 22 of these studies per year (2 studies per researcher per year), thus giving us a total of 3960 over the course of the project (covering protocols 1 and 2).

Sample sizes are determined using power analysis, typically setting a significance level of 5% and a power of 80%. However, data will be evaluated on a case-by-case basis through ongoing consultation with our biostatisticians to design the most suitable experimental approach. Based on our current calculations, group sizes will generally consist of 6 mice per treatment group. We will use one-way ANOVA to compare the magnitude of drug/nanoparticle/cargo biodistribution or vaccine response across different groups, with post hoc Bonferroni correction for multiple comparisons. A p-value of less than 0.05 will be considered statistically significant.

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

In these and larger studies, good experimental design will ensure the combination of control groups and the comparison of multiple experimental entities or doses (or where appropriate, the utilisation of factorial experimental design).

The work conducted within these studies is based on improvement of formulations therefore control groups will be restricted to benchmark groups so that improvements can be tested and negative controls will be avoided unless absolutely necessary. All studies will be built on pilot data from either small group trials (n of 1 to 3) or based on previous data generated.

Advice on group numbers with reference to the data specific to our experiments will be obtained from biostatistician/s experienced in statistics relating to animal experiments and peer review of proposed animal work.

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

IVIS/CT is an alternative to performing biodistribution studies using radiolabelling of the administered substance. Because the imaging is performed under anaesthesia, the same animal can be used throughout the study, rather than having to euthanize animals at each

analysis time point. In the long run, this can significantly reduce the number of animals used.

We will also only use animals when the lead compound shows activity in in-vitro tests.

We have investigated the role of biological sex in our mouse studies and have demonstrated that in biodistribution and protein expression, biological sex has no impact, allowing us to use either biological sex. However, we have seen an impact for vaccine studies, and we have adapted our models based on this. We have also developed a pre-screening model for liposomal adjuvants where we backcorrelated in vivo data to in vitro data, and this has allowed us to reduce the number of formulations that progress into preclinical 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.

For reasons mentioned above, the mouse model is the most widely used model for initial vaccine immunogenicity evaluation and the initial in vivo assessment of developmental new drugs and delivery systems or formulations. The use of the mouse model enables cross comparison with other studies and hence more widely applicable results and context within which such results can be evaluated. We will draw on previous experience, latest research and best practice to ensure doses of substances and dosing techniques are optimal and we will set the earliest possible end point for the experiments. The least invasive dosing route and schedule will be used whenever possible.

Similarly, in terms of drug delivery and biodistribution, a large number of studies have utilised the mouse and rat models and therefore evaluation and comparison of the results across the literature is possible. The rat model may be appropriate only where it is deemed that the murine model may not generate sufficient data (e.g., in some biodistribution studies) or where there is a need for cross comparison with other studies. As for protocol 1, we will follow best practice guidelines for dosing and imaging, aiming to minimise any pain/discomfort and to shorten the duration and number of imaging sessions.

Protocols will be performed under the Mild (protocol 1) or Moderate (protocol 2) prospective severity limit and animals will be monitored closely to ensure they do not go beyond these.

For studies involving the use of restrainers, mice will be habituated to the restrainer before the study begins to minimise stress of the animal. Where time allows, we would aim to handle the mice before studies to get them used to handling.

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

The immune systems and physiology of lower vertebrates are significantly different from humans, including restricted antibody diversity that may be partly because some

immunoglobulin segmental elements do not undergo genetic rearrangement in the same way as that which is seen in humans (and that is also seen in mice). In addition, for biodistribution studies and drug delivery, the murine model may be the model of choice for these studies as mouse physiology, in terms of organs and systems, has many parallels to human systems.

At present, it is impossible to recreate the biological environment in terms of biodistribution throughout complex physiological systems following the administration of a therapeutic or prophylactic agent. For this and the evaluation of biological effects in some systems (such as the immune system mentioned here), rodent testing may be necessary.

Rats will only be used where the mouse model is not adequate to supply the necessary quantities of blood required for analysis or where the murine model would not serve as an appropriate comparison point for a study.

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

As mentioned above, we will do this by: using best practice in terms of dosing, selecting the mildest possible route for dosing that is appropriate for each study/formulation, aiming to reduce as much as possible the number of interventions and length of studies, using habituation techniques to reduce stress and positive reinforcement to minimise aversive behaviours.

We have now acquired sufficient data and expertise in IVIS to complete the majority of our Protocol 2 experiments in a reduced timeframe of 48 hours, thus using fewer imaging sessions.

Based on previous publications, we will not use Freund’s complete adjuvant as a vaccine adjuvant control due to its potential for causing distress to animals.

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

The establishment’s good practice guidelines for dosing and imaging, the NC3Rs dosing guidelines; plus many of the excellent 3Hs Initiative recommendations.

World Health Organisation (WHO) and the European Medicines Agency (EMEA) also offer extensive guidelines on parameters for non-clinical assessment of vaccines.

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

By keeping abreast of the relevant literature, checking the NC3R's website and relying on the expertise of animal care staff, NACWO, NVS and NIO, the latter of which shares targeted advances with research groups. Our group have an excellent relationship and communication with Named Persons, our 3Rs Champion and the animal care staff at our facility. Hence, we are confident of being able to incorporate relevant 3Rs advances to our work as they come up.

World Health Organisation (WHO) and the European Medicines Agency (EMEA) also offer extensive guidelines on parameters for non-clinical assessment of vaccines.

20. Peripheral gate in somatosensory system II

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

nociception, pain, sensory neuron, ion channels, excitability

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 discover mechanisms of gating of pain-related information at the peripheral nerves.

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 programme of work may lead to the development of improved, novel means by which both acute and chronic pain can be treated. By designing pain treatments, that act locally, only at the affected nerves, it may be possible to avoid side effects in the brain. The main drawback of the major current analgesics, such as opioids, is that they act in the brain and affect not only pain, but other brain functions. These ‘side-effects’ may result in tolerance, addiction and other significant clinical problems. Re-tuning a ‘painful’ nerve should not be liable to such side-effects. The economic costs associated of chronic pain reach hundreds of billions annually. Enabling individuals to return to work sooner or avoid absences altogether through innovative pain control and treatment approaches can significantly boost the national economy and enhance international competitiveness, ultimately improving the overall quality of life for individuals.

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

Our main outputs are the following.

Scientific knowledge dissimilated in research publications, conference presentations, research seminars, as well as through research data depository

Computational models, analysis algorithms & code

Grant applications

Some outcomes could lead to the patentable IP, but this is not the typical outcome of this research

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

We believe that the evidence emerging from this research will shed light on the mechanisms of peripheral processing of pain, including chronic pain; main beneficiaries of this research programme are the following.

Pain scientists, e.g. those involved with the UKRI Advanced Pain Discovery Platform (APDP) aiming to “break through the complexity of pain and reveal potential new treatment approaches to address a wide spectrum of chronic and debilitating clinical conditions”.

Neuropharmacologists, as we will identify new druggable targets for pain.

Researchers of other medical areas. Several pathologies, including diabetes and osteoarthritis, are associated with pain, so our impact will reach to these areas of biomedical sciences.

International & industrial partners. This collaborative project will strengthens existing links with US, China and industry.

In the longer-term, people suffering from chronic pain will benefit from any therapies arising from this research

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

This research is naturally collaborative and includes funded collaborations within the UK and abroad. Thus, we have funded collaborations with King's College London, University of Texas at San Antonio, USA and Hebei Medical University (China).

We are sharing our data, analyses and code via research depositories, such as Research Data Leeds; GitHub.

We are keen on the dissemination of scientific progress to a wider audience. Aside of scientific publications presentations, our results will be disseminated through the universities’ websites (e.g. https://neural.leeds.ac.uk/) and, periodically, highlighted in wider media (example: https://www.metrionbiosciences.com/an-interview-with-professor-nikita-gamper/).

Species and numbers of animals expected to be used

- Mice: 4100
- Rats: 2100

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 and rats as these are widely accepted species for conducting studies on the nervous system. There is a broad literature available to compare our findings with and all our previous animal studies were conducted in rats and mice. Mice are a first species choice for studies including genetic manipulations due to the availability of tools, resources and a large collection of ready-made transgenic mouse strains. Rats are larger animals, which makes it easy to access the nerves we are studying.

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

Typically animals will be used to investigate how information related to pain is processed within peripheral nerves. The experiments will involve surgical access to peripheral nerves for the application of stimuli and recording of nerve activity. The animals may undergo genetic manipulation before or during such measurements/stimulation. These manipulations are used to change expression of specific genes in order to learn about the role of these in pain processing. In some animals, a well-accepted models of chronic pain (such as neuropathy or pain due to tissue inflammation) will be established before any manipulations or measurements of peripheral nerve activity. Our experimental strategy can be exemplified in the following typical experiment: mice with a gene of interest genetically deleted and ‘normal’ mice will receive identical lesions to a peripheral nerve to create a neuropathy. We then will assess if the neuropathic pain is altered by the gene deletion by comparing behavioural manifestations of neuropathic pain between the two types of mice. We will also measure and compare various parameters of nerve function between the two types of mice.

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

Adverse effects will be closely monitored. Adverse effects of administration of substances may include lethargy, convulsion, starry coat, weight loss.

Surgical procedures performed under this license carry low risk of cardiac arrest or other anaesthetic complications. In previous similar experiments conducted by us, anaesthetic complications were rare (less than 1%). These will be minimised by providing stable anaesthesia.

On recovery from surgery, animals may experience minor discomfort from the cutaneous incision wound and post-operative analgesia will be used at the time of surgery and post operatively.

Mild (short-term, low-intensity) pain or moderate (short-term moderate, or long-lasting mild) pain may be a consequence of the use of chronic pain models (such as peripheral nerve injury or inflammation). Additionally, motor effects causing gait deficit or lameness may also develop. Similar deficits can develop following implantation of devices delivering stimuli to the sensory/motor neural pathways.

In rare cases implanted devices may produce inflammation.

Animals will be inspected twice daily and weighed at appropriate intervals (at least twice a week) by licensees and trained animal care staff for signs of excessive distress and ill health. These signs may include > 10 % body weight loss, decreased grooming behaviour, biting of affected or associated areas, ataxia, increased aggression and disturbance of the sleep/awake cycle. If any of these signs persist for 24 hrs, or in case if signs of distress are severe and continuous and preventing normal behaviour, the animal will be humanely killed and advice of the Named Veterinary Surgeon will be sought in order to establish possible causes and prevent recurrence.

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)?

Some of the experiments (pain models) will be of mild or moderate severity. Lesions to peripheral nerves or peripheral inflammation may result in moderate pain and/or hyperalgesia and in some distress associated with it. We estimate that the following breakdown:

Moderate severity - 25%

Mild severity - 10%

Subthreshold severity - 65%

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?

Pain management is an unmet clinical need as many types of pain (i.e. neuropathic pain) cannot be successfully treated with current medications. As pain is an experience carrying both sensory and emotional aspects, there is currently no in vitro or in silico model system capable of recapitulating pain in it's entirety. Therefore, experiments with mammals are necessary.

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

We are using several alternatives listed below:

Expression systems to investigate functions of individual pain-related molecules.

Computational modelling to investigate biophysical processes underlying pain signals.

Why were they not suitable?

Both the above approaches are valuable but the results obtained with these still need to be verified in vivo, before a conclusion about real contribution to pain processing of a

molecule or phenomenon of interest could be made. These alternative approaches do help to reduce animal testing by identifying the most plausible hits.

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 calculated on the basis of previous projects with similar remit and methodology conducted in the applicant's laboratory.

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

In all the in vivo experiments, we will keep the group size to a minimum sufficient to detect significant changes between the groups or conditions. This project is closely aligned with the 5-year programme grant awarded to the applicant by the Medical Research Council and uses a common experimental design approach. NC3R's Experimental Design Assistant has been used for the animal group size calculations.

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

Our aim is to reduce the number of animal experiments whenever possible. A large share of our experiments is done with cultured neurons and glial cells. This is a very efficient way of animal usage since a culture from one animal usually provides enough material for a week of experiments or even longer. Mathematical modelling will also be extensively used to reduce animal 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.

We will only use pain models that are well established in the field. In most cases in these models animals only experience relatively mild distress, close to the threshold of feeling discomfort. As animals are checked daily, signs of significant discomfort will result in immediate sacrifice of the animal with humane schedule 1 procedure.

In some experiments we will implant devices that allow stimulation of, application of substances to and/or recording from peripheral nerves and ganglia. All these approaches have been published by us and others in journals with high ethical standards for animal

research (such as Pain, Journal of Clinical Investigation etc.) and scrutinised during the review process.

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

We are conscious about refinement of our methods towards a possibility to use less sentient animals. A good example of our approach is introduction of decerebration step in some protocols; this procedure eliminates the possibility of accidental recovery from anaesthesia during the experiment and, thus, reduces potential suffering. This refinement has been introduced by us as an amendment during the previous PPL period.

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 pain or distress, a validated scoring systems will be used to objectively evaluate their well-being.

Group housing will be used whenever possible. Social interaction can reduce stress and improve welfare.

Analgesics will be used post-operatively to reduce pain.

All team members conducting animal experiments will be trained to handle animals gently and minimize stress during procedures. We will foster a culture of compassion and respect for animals within the research team.

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

We will closely follow the UK home office and NC3Rs guidelines (such as ARRIVE). Below are some example resources that we will use for guidance:

https://www.gov.uk/guidance/research-and-testing-using-animals
https://nc3rs.org.uk/arrive-guidelines https://nc3rs.org.uk/3rs-resources

https://www.ukri.org/who-we-are/mrc/our-policies-and-standards/research/research-involvinganimals/3rs/

https://www.ukri.org/who-we-are/epsrc/our-policies-and-standards/policy-on-use-of-animals-inresearch/

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

The project team will stay updated via

Research publications related to the 3Rs and new methods, technologies, and best practices

NC3Rs website iii) Attending conferences, workshops, and webinars focused on the 3Rs iv) Networking with colleagues to learn best practices

Implementing 3R advances in this project will involve initial evaluation of feasibility, cost, and impact, followed by ethical approval and (if necessary) seeking amendments to the current license.

A good example to this approach is introduction of decerebration step to our protocols during previous

21. Role of steroid-producing immune cells in inflammation, immunity and 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
    - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants

Key words

steroids, immune regulation, cancer, inflammation, immunotherapy

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?

Recently, we discovered that immune cells can produce steroid hormones (Steroid hormones: A group of biological chemicals mainly secreted by “steroid glands” such as adrenal glands, testes, and ovaries). This project aims to discover the basic principles of immune cell-mediated steroid hormone production, and their role in regulating infection, inflammation, and cancer, as well as to exploit this newly created knowledge to develop new therapeutic 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?

Immune cells are required to fight infections by bacteria and viruses. However, their disordered or dysregulated function causes many disease processes. When immune cells

attack the own body, it is called autoimmunity. In allergy, immune cells respond to nonharmful substances and show pathology. When immune cells fail to recognise and kill cancer cells, it leads to tumour progression. The immune system comprises diverse cell types that can promote or inhibit inflammation and immune responses as required. Suppressive or inhibitory components of the immune system are required to restrict excessive or uncontrolled immune reactions. However, if maladapted, they can suppress potentially beneficial immune responses. Such maladaptation is known as immunosuppression

(Immunosuppression: Suppression of the immune system). Immune reaction and immune suppression are targets for the development of new drugs.

Newly developed therapies targeting mechanisms that suppress immune cell function (i.e., immunosuppression) have shown promise in cancer patients. These drugs unleash the power of immune cells and help kill cancer cells. However, the knowledge of immunosuppression mechanisms is limited. In this study, we will create new knowledge by revealing the basic principles of one such immunosuppression mechanism. We discovered that certain types of immune cells produce steroid hormones. These steroid-producing cells suppress immune responses. In cancer, they suppress antitumour immune responses. Steroid hormones are a group of biological chemicals mainly secreted by “steroid glands” such as adrenal glands, testes, and ovaries. Steroid hormones regulate immune cell function. In general, they are immunosuppressive and anti-inflammatory. We will investigate how immune cell-produced steroids regulate cancer, inflammation, and immunity. The study should lead us to discover new therapeutic targets and therapeutic agents against cancer. Modulation of these steroid-producing cells would benefit certain types of cancer treatment and inflammatory diseases like allergies and asthma.

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

On a global scale, a common practice in the clinic is to target immune cell function as a therapeutic approach to bring health benefits. For example, therapies targeting immunosuppressive mechanisms in cancer are presently revolutionising the treatment for patients with metastatic disease. This type of approach unleashes the power of the body's immunity against cancer. By contrast, anti-inflammatory and immunosuppressive drugs like steroids are used to treat allergic asthma, allergies, and autoimmunity.

This study will extend our basic knowledge of how the immune cells' function is controlled or regulated. In particular, we will learn how steroid-producing immune cells and their secreted steroids regulate immune cell function during inflammation and immune reactions, including cancer. We will identify and confirm new therapeutic targets for inflammatory and immune dysfunction-related diseases such as cancer, allergies and asthma. We will publish our research in peer-reviewed journals and present our findings as oral presentations and posters at national and international conferences. The research will also provide a basis for the development of new therapies aimed at controlling immune function in patients with a variety of disorders in which the immune system plays a critical role in inflammatory diseases, infection, and cancer. More specifically, we will demonstrate that intervening immune cell steroid signalling may bring benefits for cancer-associated inflammation and immunity. At the same time, we will show evidence that augmenting immune cell steroid signalling may bring benefits for inflammation and allergic asthma-related disorders. We will develop new therapeutic immune cells and test their efficacy in mice models. These therapeutic cells could be used in treating cancer and inflammatory diseases. These novel products with therapeutic potential will be patented.

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

The project will benefit researchers in related scientific fields, such as cancer immunology, inflammatory disease biology and infectious diseases. The work is relevant to scientists aiming to develop new therapies for individuals with immune-mediated disorders, cancer, and infection, including pre-clinical researchers and the pharmaceutical industry. The impact of these diseases and disorders on human life is awfully daunting. According to the World Health Organisation (WHO), **cancer is a leading cause of death worldwide**, accounting for nearly 10 million deaths in 2020, only. The most common in 2020 (in terms of new cases of cancer) were: breast (2.26 million cases); lung (2.21 million cases); colon and rectum (1.93 million cases); prostate (1.41 million cases); skin (non-melanoma) (1.20 million cases); and stomach (1.09 million cases). The most common causes of cancer death in 2020 were: lung (1.80 million deaths); colon and rectum (916 000 deaths); liver (830 000 deaths); stomach (769 000 deaths); and breast (685 000 deaths). Each year, approximately 400,000 children develop cancer. The most common cancers vary between countries. Our research suggests that steroid-producing immune cells are involved with all types of solid tumours tested, however, not all patients show this mechanism of immunosuppression. It warrants further studies. In the future these patients will be benefitted. No statistics are available on how many researchers are involved; however, the number must be big.

WHO reported that **Asthma** affected an estimated 262 million people in 2019 and caused 455,000 deaths. Other **inflammatory diseases**, such as allergies, are a big burden to healthcare and a major topic in immunology and medical research. This project is expected to bring benefits to these patients in the future.

As **this project will identify and confirm therapeutic targets of cancers and inflammatory diseases (such as colitis, colitis-driven cancer, allergies and allergic asthma)** and virus infection, in the long term, the knowledge may bring patients benefits from the above-mentioned diseases. To expedite and amplify this possibility, we will immediately share the research results with the community. Whoever is involved with the research on these areas will benefit.

In addition, the intellectual property generated by this study might be commercialised in the future.

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

The outputs of this project will be disseminated through research publications and presentations. Data and reagents will be directly shared with academic and industrial sector researchers ahead of publication. We collaborate with multiple research groups of the field. We also strive to engage the public with our science. We will secure intellectual property and commercialise our research to foster UK industrial and bioscience growth.

The unsuccessful approaches will be submitted and pre-printed on bioRxiv (https://www.biorxiv.org/), an online archive and distribution service, for sharing with global scientists. We will also communicate these results for publication in peer-reviewed journals.

Species and numbers of animals expected to be used

- Mice: 28,330

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 vitro (in a test tube, culture dish, or elsewhere outside a living organism) reproduction (modelling) of the immune reactions (interactions between immune cells and with the inflamed tissue) is extremely difficult. It often does not reproduce what happens in a living organism. This is because of the complexity of the immune system. Immune responses are dynamic interactions between multiple cell types and tissue types. In other words, immune response is an integrated physiologic process. The immune system in mice is similar enough to the immune system in humans. Many genetically modified mice and reagents are available to study the immune system. Therefore, this species can be used more efficiently than any other species to study the role of genes that regulate the immune system.

In general, we usually use adult mice. The immune system of adult mice is similar to that of humans in many ways. Our research focuses on diseases which primarily affect adults. A similar is true for the endocrine system. The endocrine system is made up of glands that make hormones. Hormones are the body's chemical messengers. They carry information and instructions from one set of cells to another. The endocrine system influences almost every cell (including immune cells) of our body. In adults, the endocrine system is stable and well-developed. We will use mice of similar age to reduce the variability of our experiments. Apart from studying adult mice, we will study aged mice in a few experiments to investigate the effect of ageing. We discovered this new fact: Immune cells can produce steroid hormones. Mice, adult and aged, would help us understand the role of these steroidproducing immune cells.

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

Most of the mice will be used in breeding genetically modified animals. We will use two types of genetically modified mice. Most of the genetic modifications are in the immune cells (that regulate immune cell function). Some of the mice would have genetic mutations that cause cancer development. These genetic modifications will either help identify those cells or be used to test the effect on immune cells' regulation. These genetically modified mice will be used in experiments. All the mice will be killed in a humane way, and the organs related to immune cell function will be analysed in the laboratory. These mice will be used in experiments to test how the immune system responds to infections, inflammation, and cancer. Typically, animals will experience mild, transient pain and no lasting harm from the administration of substances by injection using standard routes (into the tail vein, under the skin, into the peritoneal cavity).

Most of the experimental mice will be used to test the immune response against tumours. We will implant small numbers of tumour cells under the skin (i.e., subcutaneous injection) of animals and let them grow. To test the effect of a gene, we will use mice with genetic alterations in the immune system to tumours. Tumour growth will be monitored regularly by trained staff. Animals will not be allowed to suffer excessively, or beyond well-defined criteria. Animals that are likely to develop adverse effects that extend beyond the well-defined limits will be killed using a humane method before they reach that point. After humanely killing animals, tumours and organs, such as spleens, lymph nodes and lungs, will be taken and analysed in the laboratory to gain insights into how immune cells

respond tumours. The conclusion of the experiment will be how a specific gene controls immunity against tumour. In general, these experiments take around three to four weeks from tumour injection to humane killing. Animals may receive substances (known drugs or potential drugs) during tumour growth to examine the therapeutic potential. Similarly, we will test animals' immune responses against experimental diseases (infections and inflammations). The typical duration of such disease models is up to 16 weeks.

We also need to use models of inflammation-induced cancer, infection, asthma and allergic inflammation to test the role of steroid-producing immune cells, in a very limited number of experiments. In a few experiments, experimental mice will have received therapeutic immune cells derived from another mouse or immune cells with known effector function generated outside of the body (in sterile Petri dishes or flasks). In such experiments, animals will typically receive an injection of immune cells in the tail vein and then infection or inflammation or cancer will be induced. To induce inflammation or allergy or cancers, well-established substances/agents will be administered via any one of the appropriate routes: via nose inhalation, via mouth with food or drinking water, into or under the skin by injection, into the tail vein, into the peritoneal cavity, and rectal route.

Animals will be routinely assessed for signs of illness and weight loss. Animals likely to breach the severity limits of the protocol will be killed humanely.

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

Most of the animals will be used in breeding to expand genetically altered mice, which is essential for the research and will experience no significant adverse effects. Adverse effects from experimental procedures depend on what experimental model is being used. For example, likely adverse effects in cancer models include the ulceration of tumours and skin damage over the site of injected tumour cells. Ulceration triggers a decision to the humane killing of the animal. Mice may show breathing problems in the lung tumour model. Mice with signs of laboured breathing will be humanely killed. Mice may lose weight. Mice with 15% weight loss will be humanely killed. Experimental animals in the colitis model may lose weight and exhibit reduced activity. Animals with colitis are expected to have diarrhoea. Blood is occasionally observed in the stool. Mice will be humanely killed if their weight loss reaches 15%, or if they show clear signs of diarrhoea and rectal bleeding that does not resolve within 24 hours. Each cycle of the colitis model (in the chronic colitis model) persists for 3 to 5 days with a minimum gap of two weeks that helps them recover. Administration of influenza virus, on a maximum of two occasions at least two weeks apart, may cause body weight loss but does not prevent normal feeding, drinking, or other normal activities. Colitis induces tumour formation. In colitis-driven tumour models, administering colitis and tumour-inducing agents is expected to result in intestinal inflammation and weight loss. Any mice that reach 15% weight loss will be humanely killed. In the asthma model, the mice are expected to have trouble breathing. In the skin inflammation/allergy model, localised skin itching, discomfort, mild pain, and skin scratching and picking (localised at inflamed area) behaviour are expected. However, reactions are expected to be highly localised to the area of topical administration and the induction of a whole-body symptoms is not expected. Skin inflammation (atopic dermatitis) model typically takes up to 1 month and involves repeated (no greater than 10) topical administrations of a chemical that induces skin inflammation (dermatitis), usually on both surfaces of the pinna of one ear. Animals will receive substances, such as antibodies and steroid hormones, prior to or during the induction of inflammation. During or after the

resolution of inflammation, animals will be humanely killed, and their tissues will be analysed in the laboratory.

A limited number of NSG mice (NOD scid gamma mice) will be used. These mice are deficient in many of the immune cell types. An estimated 20% (or less) of mice from these NSG mice may show swelling around the hocks. Any animals experiencing swelling around their hocks may be given an altered enrichment, pain relief and/or anti-inflammatories in consultation with the NACWO and/or 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)?

Mice mild 80% moderate 20%

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?

Modelling human diseases and related immune reactions in animal models is necessary to understand how our immune system works. Several crucial interactions of immune cells with other cells and substances in living animals cannot yet be generated (modelled or reproduced) in test tubes or cell culture dishes. Immune cells are distributed throughout the body (in different organs). They are able to migrate into almost all body tissues to protect the body from infection and cancer. These features can not be reproduced artificially. Therefore it is essential to investigate immune cell behaviour and function in the whole animal. Adaptive immunity-related immune cell function (the type of immunity which remembers previous exposures of antigens, for example, how a vaccine works), which is the subject of this research, evolved in vertebrate animals and is not present in less sentient organisms. Many cell and biological molecule-related features among vertebrates are highly conserved between mice and humans. Many useful tools and well-established models for experiments in mice already exist, allowing us to perform research using mice in an efficient manner that minimises the number of animals we need to use. Therefore, the use of mice in this research is necessary.

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

We considered computer-based (computational) analyses. These coputer-based approaches (i.e., bioinformatics and cheminformatics approaches) use publicly available data and predict molecular and cellular interactions. We also considered cell cultured-based experiments that are done in Petri dishes and test tubes for certain molecular pathways that have an important role in controlling the immune system. For example, undertaking a computational approach of drug screening and drug repurposing study, we identified novel drugs that are already approved but can be repurposed for cancer treatment. These drugs modulate the immune cell-mediated steroid production and action

of immune cellproduced steroids. To test the efficacy of those drugs, some cell culture-based experiments were sufficient to make a conclusion for our biological questions. However, many key questions (the most important questions) remain unanswered from such experimental approaches. For those animal experiments are essential. For example, whether predicted cell-to-cell and molecular interactions are really happening inside the body of an animal. We used steroid-producing cell lines and laboratorygenerated steroid-producing cells. We use human patients' samples on a regular basis to check the presence of steroids within the tissue, gene expressions that are involved with steroid production and steroid action, and proteins that are involved with steroid-mediated cancer progression. We use human blood obtained from healthy donors and patients to test the role of immune cell-mediated steroid production and the effect of those steroids on human immune cell functions. To fulfil the same purpose, we also use several cancer and immune cell lines. We engineer human blood-derived immune cells to generate genetically engineered versions that can be used as therapeutic cells in the future.

Altogether, these alternative approaches replaced at least double the number of mice needed to test directly in mice models.

However, there are unanswered questions related to how the immune system works within the body and for what mice work is most suitable but with the least suffering and pain.

Why were they not suitable?

Computational analyses, test tubes or cell culture-based experiments using purified immune cells fail to reveal the various interactions of immune cells with other cells and substances in the body. Moreover, organs and tissues contain complex mixtures of both immune and non-immune cells, each of which can signal to other immune cells and affect their behaviour. Reproducing such a living tissue set-up is impossible at present. Insights into whether specific components of the immune system can be targeted to improve therapeutic outcomes are also difficult to gain using experiments performed in test tubes or culture dishes. We will perform two types of animal experiments that are impossible to model in the culture dishes or test tubes. First, the role of steroid-producing immune cells in different animal models of diseases (mainly cancer, but also in asthma, allergic inflammations, and colitis). In particular, we will validate (confirm) the predictions that are derived from computational analysis and test tube or cell culture-based studies. Second, we will modulate (induce or reduce) the immune cellmediated steroid production and steroid action, genetically or pharmacologically, to test whether such modulation can be used as a therapeutic strategy. Before arranging clinical trials in human patients, such a preclinical experimental approach in a limited number of mice would be necessary to demonstrate the proof of the principle. Reproduction of such pathological situations and disease outcomes because of genetic and pharmacologic targeting of immune cell steroid production is impossible in test tube or cell culture-based 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 provided the estimated number of mice to be used under each protocol of the license. The use of littermate controls is a requirement in our experiments to ensure an identical environmental and genetic background between experimental and control animals in experiments. We calculated the number of mice required per line to produce sufficient experimental and control animals from heterozygous (consisting of different alleles of particular genes) crosses to allow for experiments to be conducted with adequate statistical power. We used statistical tools that are designed to help researchers find the least number of animals. The calculated numbers are sufficient for the generation of genotyped control and experimental animals at a typical age (range 8-12 weeks) at which they can be used in experiments. Our calculations are based on average litter sizes of 6, an average time between litters of 6 weeks, an expected sex ratio of 0.5 and an expected Mendelian ratio of 0.25 for experimental and control progeny. All experimental mice will be a continued used from the protocol 4 (i.e., breeding and 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?

Experimental control groups are important for most of the experimental models being used and provide a means to improve data quality from our experiments involving animals. The generation of high quality data eventually provides a means of reduction since definitive results will prevent the necessity to perform experiments again, either due to technical failure or a lack of broad applicability/interpretability to other researchers in the field. We use resources available for designing experiments with an appropriate randomisation and blinding strategy and sufficient sample sizes to enable adequate statistical power, such as the NC3Rs experimental design assistant

(https://www.nc3rs.org.uk/experimental-design-assistant-eda). Many similar experiments have been done before in collaboration with our collaborators. We used those experiments and cohort size as benchmarks. We use online available experimental designing tools, which are designed to minimise the number of animals used in experiments but sufficient to draw conclusions from the results.

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 monitor the breeding performance of our mice to ensure that the minimum numbers of animals are used. We use colony management software that helps optimum production and avoid overproduction. The use of inbred strains helps to model experiments with definitive conclusions using minimally required mice. The strategy minimizes variation and allows us to robustly ascribe the cause of phenotypes observed to the introduced genetic mutation. Our research will require the generation and maintenance of complex multiallelic mouse genetic strains at colony sizes sufficient to provide littermate experimental and control animals from heterozygous mating. Our calculations were based on previous experience generating and maintaining multiallelic mouse strains obtained from our collaborators who perform similar experiments.

The maintenance of specific pathogen-free health status and controlled environment will reduce experimental variability and decrease the cohort size required for sufficiently powered statistical analysis. Members of the team receive training in statistics used in our experiments. Biological plausibility, mechanistic insight and consequences of the effect will inform the interpretation of significance in addition to obtained statistical significance values. Studies will be reported according to ARRIVE guidelines.

We keep ourselves up to date with the literature for methods to reduce the numbers of animals required and to improve experimental design. If a suitable allele has been generated elsewhere, we import this by embryo or sperm transfer, or by shipping live mice where sperm or embryos are unavailable, and inter-crossing the offspring to obtain the required genotype. Cryopreservation and rederivation by embryo transfer are important for both the quality of the science and for a reduction in the number of animals bred.

Genome editing technologies such as CRISPR/Cas9 mediated gene editing offer a potential for reduction. CRISPR/Cas9 allows the function of genes to be tested without germline mutagenesis and the establishment of mouse colonies to test gene function. We have started to develop powerful new high-throughput CRISPR/Cas9 screening approaches to identify functionally relevant genes within specific immune cell types in vitro. While not relevant to a substantial proportion of our present

research, this approach has the potential to lessen our need to perform preliminary testing of gene function using germline mutagenesis with subsequent reduction in mouse 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.

We will minimise suffering by carefully observing the mice undergoing procedures and adhering to AWERB handbook guidelines. Following the guidelines, we will assess clinical signs that may trigger the end of an experiment for a particular mouse or a cohort. We will choose the model which causes the least suffering to animals but is sufficient to answer a specific question.

We prioritize using mice that have become singly housed, as soon as possible. In consultation with animal technicians and veterinary staff, we modify protocols and introduce refinements to the protocols and animal husbandry that reduce harm. This includes alternative bedding for animals with reduced mobility, providing access to food in gel format, use of analgesics and more frequent monitoring for mice at increased risk.

Where tumour immune responses are being tested, we will primarily implant tumours by subcutaneous injection. This model minimises animal suffering by enabling non-surgical introduction by needle injection of tumour cells into animals. Further, the subcutaneous space provides a compliant space for tumour growth resulting in minimal suffering to mice as tumours develop. In general, subcutaneous or intraperitoneal injection of tumour cells is preferable from an animal welfare perspective to surgically implanting cells into the internal organs in recipient animals. Therefore, subcutaneous tumour models form the mainstay of the proposed work involving tumour immunity, although non-surgically implantable locations will also be used, such as melanoma tumours injected into the intradermal space, and breast carcinoma cells injected into the mammary fat pad, ovarian cancer cells into the peritoneal cavity.

Infection with the influenza virus will be carefully controlled to minimise adverse effects by using the least harmful strain and minimum effective dosage (as reported by previous studies). Batches of infectious agents will be standardised using in vitro assays, and the lowest dose will be sufficient to elicit the required immune response given. Similar strategies have been taken for all inflammationrelated protocols where the choice of inflammatory agents and their dosages are kept that is minimally required. Additionally, we have established clear humane endpoints for all protocols to avoid unnecessary suffering of infected animals.

Perioperative and post-operative analgesia will be given when necessary, using advice from the NACWO and NVS. The choice of analgesic, duration and dose will be adjusted to the clinical signs observed considering possible impacts on the experimental plan. Perioperative analgesia will be given and maintained after surgery for as long as is necessary to alleviate pain. Analgesia will also be administered to control pain for painful procedures. In addition, procedures are refined to reduce the suffering they cause and training of staff conducting procedures is maintained to minimise interoperator variation and suffering.

Administration of tamoxifen in the diet is known to cause neophobic effects in mice. We have gained experience from our collaborators and information from our project support team that has enabled us to make refinements to our tamoxifen administration protocol reducing adverse events related to tamoxifen-induced neophobia substantially.

Any animals experiencing swelling around their hocks may be given an altered enrichment, pain relief and/or anti-inflammatories in consultation with the NACWO and/or NVS.

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

Lesser model organisms, such as zebrafish, have far less conserved immune systems to humans than mice, are less well characterised and many of the tools used to study immune function in mice have not been generated for lesser species. Thus, while we have considered other ways to reduce and refine our use of animal models, our funded research programme would be impossible to complete using less sentient species than mice. Access to primary and secondary lymphoid organs and peripheral tissues, such as the lungs, gut, spleen and lymph nodes, is required for the proposed analyses. Such access is limited in human studies. Adoptive transfer experiments are also possible using inbred mouse strains since they are genetically identical and do not express mismatched antigens for graft rejection.

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

We constantly refine our experimental techniques to prevent unexpected adverse events, minimise variability and thereby reduce experimental group sizes, and minimise suffering caused to animals by working with small groups of animal technicians longitudinally on specific projects so that experience of experimental techniques builds over time, and by producing and refining standard operating procedures for interventions such as cell injection and tumour measurement.

We monitor animals daily. We will keep close contact with animal technicians to increase the monitoring frequency and measurements when necessary and humanely kill animals once any significant clinical signs show. We will continue to enhance this practice.

To reduce the pain caused by administering any immunoregulatory substances, we will seek advice from animal technicians, NACWOs and NVSs.

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

We will follow these guidelines below:

Prescott MJ, Lidster K (2017) Improving quality of science through better animal welfare: the NC3Rs strategy.

Lab Animal 46(4):152-156. doi:10.1038/laban.1217

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 AJ, Clutton RE, Lilley E, Hansen KEA, Brattelid T (2018)

PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) Guidelines

ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines

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

We will have regular discussions with the researchers, animal facility technicians, and managers at our institution to review current approaches and whether there are any new 3Rs.

Research staff will subscribe to the NC3Rs e-newsletter, providing updates on updates to 3Rs principles and methods, funding opportunities, 3Rs events and publications.

We will encourage staff to attend NC3Rs events and workshops as a way of keeping abreast of 3Rs advances and approaches.

We will contact NVSs (Named Veterinary Surgeons), NACWOs (Named Animal Care and Welfare Officers) and animal technicians to seek advice when necessary.

22. Red deer space use in a time of rapidly changing habitat management

Project duration

5 years 0 months

Project purpose

- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Animal behaviour, Animal movement, Disturbance, Ecology, Reproduction

Animal types	Life stages
Red deer	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?

We aim to collect in-depth data on the activity and movements of red deer, simultaneously with high quality data on aspects of the environment that could influence those. By doing so, we aim to identify the impacts of management and land use on red deer, and to provide insights into improved management of deer, promoting cooperation between landowners with conflicting management goals and other users of the landscape.

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?

Management of the Scottish Uplands is changing rapidly. Ambitious goals for carbon neutrality ("net zero") promote reafforestation and peatland conservation, whilst the biodiversity crisis and policies such as 'biodiversity net gain' promote rewilding. These land uses compete with traditional forms of agriculture and other pillars of the rural economy, including red deer stalking. A particular area of dispute is around appropriate red deer densities. Deer control to reduce their numbers is commonly conducted in areas where the focus is on rewilding or reafforestation. By contrast, estates with economic interests in deer stalking are keen to maintain relatively high abundances of deer. This can lead to

source-sink dynamics, whereby deer move into areas newly cleared of deer by intensive culling activity. That is, deer move from where they are wanted to where they are not wanted. That, in turn, presents grounds for conflict. Other aspects of management, such as supplementary feeding, fencing and tree planting can further influence deer movements, as can legitimate forms of recreational land use. At present, debates over how deer movement and space use will respond to changes in these factors have a relatively limited scientific foundation, because the movement and space use of red deer on the Scottish mainland have not been studied in detail in relation to these aspects of management. Predictive tools exist but have not been extensively tested. Here, we propose to study the movement and space use of red deer across a landscape of mixed uses, including stalking estates and areas of rewilding and tree planting. We will use these data to identify patterns of deer movement and the mechanisms driving them. This will allow us to evaluate the performance and refine the functioning of predictive tools. The project will also serve as an objective focus for collaborative working among stakeholders representing different interests around the charged issue of deer management in the Scottish Uplands. Ultimately, our findings will feed into more informed approaches to managing the land for deer, the environment, and people, by working with the biological and ecological needs of deer and the environment, and promoting sustainable land use.

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

Outputs from this project will include:

Data on the movements of individual deer across the annual cycle, showing patterns of habitat use and how deer movements and habitat use are affected by management, environment and disturbance.

Additional data on the movement, behaviour, and spatial distribution of red deer on the landscape.

Comparisons with and refinement of DeeRMAP, a tool developed for land managers to understand deer movements across the wider landscape.

Community engagement to bring stakeholders together around discussion of the findings and their implications for more collaborative land management.

GPS collars are widely used in deer studies that investigate movement and behaviour in deer, and their value for achieving these project goals is well established in the literature. Even a modest amount of data collection, owing to unexpected adverse field conditions or unusual levels of equipment failure, will substantially improve the data-driven basis for addressing management goals, focusing debate away from opinion, and towards collaborative assessment of data.

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

Current patterns of land use and management in the Scottish uplands are evolving rapidly. Ambitious goals for carbon neutrality ("net zero") promote reafforestation and peatland conservation, whilst the biodiversity crisis and policies such as 'biodiversity net gain' promote rewilding. These land uses compete with traditional forms of agriculture and other pillars of the rural economy, including deer stalking. There is also potential for conflict centred on the negative impacts of deer on forest recovery and peatland integrity, whilst disturbance caused by recreational land users is sometimes credited with driving deer away from estates where stalking is practised. These effects have implications for management activities, in addition to economic and ecological impacts. Furthermore, there

are welfare implications for wildlife under pressure from disturbance by humans. Altogether, conflict around land use and deer results in a system that is characterised by clear divides between the views of different stakeholders, and where there is much disagreement around the role and future of red deer within the system.

With this in mind, the benefits of the project will be to engage a variety of stakeholders in understanding more about the role of red deer in the ecology of the system, bringing them into the conversation through involvement in red deer tagging and observation, and through discussions around the strengths and weaknesses of the DeeRMAP software. The knowledge we gain will provide insight into the movements of red deer and the factors that affect that movement, independent of the pre-existing views of deer managers, land managers and rewilding organisations. More generally, this research is critical on both local and global scales as human activities, including recreation and largescale tree planting (in fenced off areas), encroach ever further into wildlife habitat. In Scotland, the detailed movement data gathered on deer in the study site will also help to inform deer management, filling a knowledge-gap around movement at this scale.

We anticipate that our outputs will be useful, as follows: 1) to inform management decisions, for example setting culling targets that consider the additional pressure of competing land uses, or determining the placement of tree planting projects in light of their impacts on deer space use; 2) to engage a wide range of stakeholders in working towards a common understanding of what affects deer movement, landscape use and impacts; and 3) to contribute to the wider discussion on deer management in Scotland which is commonly lacking detailed and precise information on deer movement. Long term benefits will be seen in the contribution to the development of sustainable practices for recreation and deer management to benefit wildlife and conservation, landowners, and the public.

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

Our work will lead to practical outcomes and we will seek to publish those in journals relevant to the discipline and to communicate them to stakeholders more widely through articles in industry magazines, presentations to stakeholder groups (e.g., the Association of Deer Management Groups), and community outreach events, such as the “Hill to Grill” programme in which the Affric Highlands staff are represented. In addition, detailed movement data provide a resource for behavioural ecologists with potential application to a much wider range of aspects of ecology. These include understanding the relationships between the true number of a species (its “absolute abundance”) and the number of that species recorded by survey methods (an “index” of abundance); developing models to help us to understand predatory success rates; and understanding how forager searching strategies mediate the relationship between area use and daily travel distance. All of these are active themes of research within the group and the data collected will be useful for advancing all of them.

In addition, retaining tissue from the process of ear-notching (for reidentification purposes after the study) will enable us to make that tissue available to other scientists, including those involved with disease surveillance and evaluating genetic diversity (e.g., for research into susceptibility to chronic wasting disease in deer).

Species and numbers of animals expected to be used

- Red deer: 60 (up to 30 adults and up to 30 calves)

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 aims and objectives of this study specifically relate to the movements of wild red deer, which are at the centre of significant management controversies. Alternatives, such as farmed deer, are unlikely to respond in the same way as wild deer and would fail to answer our research questions. For collaring, we will focus on adults because they are fully independent and will be able to rejoin the herd following the procedure. Females are important to monitor because they - especially those with young - are particularly vulnerable to disturbance (Recarte et al. 1998, https://doi.org/10.1016/s0376-

6357(98)00037-0). The effects of disturbance on females impacts their body condition and nutritional access which, in turn, has a determining influence on reproduction and, hence, population growth (which is one of the ultimate impacts of altered space use). Males are also important, as they are the focus of stalking activities and so are at the centre of conflicts around the economic impacts of different types of management on neighbouring estates. Finally, because our previous work has highlighted the difficulty of getting close enough to adult females to dart them safely, we will also tag calves, which typically remain with their maternal herds for at least 24 months. This less invasive process will allow us to gain data on maternal herd movements, without complete reliance on darting large samples of adult females.

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

Adult animals will be anaesthetised using a dart gun, in order to reduce stress. A collar with a GPSenabled tracking device will be fitted in line with best practice to minimise risks to the animal. We will also fit an ear tag and take an ear notch so that the animal can be recognised subsequently and not inadvertently admitted into the human food chain. The animal's recovery from anaesthesia will be assisted with a reversal agent. The whole procedure is expected to take about 45 minutes at most. Each collar will continue to collect data over a 24 month period, transmitting a subset of locations remotely to researchers via iridium satellite communication, and will be programmed to drop off the animal at the end of that time. The collar will then be retrieved using the most recently transmitted GPS location and/or the VHF tracking beacon. This will give access to the full set of data collected by the collar. Calves will be captured by hand within the first few days after birth and ear-tagged. This procedure takes less than 10 minutes. Solar tracking technology allows ear tags to collect small amounts of data from deer calves indefinitely, while other GPS and VHF options will allow for data collection over 12-24 months.

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

Expected impacts on adults include pain from the intramuscular dart injection, prior to the anaesthetic taking effect, and transient discomfort from the injections and ear notching as well as, potentially from the unfamiliar collar. We do not expect any of these transient effects to last more than 72 hours. Similarly, ear tagging of calves represents 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)?

The procedure is expected to have mild severity for 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?

The specific focus of the research is on the red deer in its natural habitat. It would not be meaningful to substitute it with another species.

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

Direct observation, pellet group (dung) surveys to indicate space use, camera trapping.

Why were they not suitable?

This project will investigate deer movement and behaviour in time and space. An equal focus on temporal variation in movements means that typical methods to survey deer spatial distribution in the landscape, such as pellet surveys, are insufficient. Given our interest, among other things, in direct, short-term responses to stimuli, as well as the spatial scale over which we are working, camera trapping also has limited value. Alternatives such as direct observations are limited by observer bias, difficulty in tracking multiple animals over large distances, potential influence of observer presence on the deer, and the amount of time required. By using GPS collars, the project limits interaction with the deer to a short capture operation, whilst maximising data quality and quantity to build a comprehensive response to our research questions.

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 guided by precedents among published studies employing the same techniques, and by our calculations of the number of animals needed to supply enough data to answer our research questions robustly.

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

We are using collars made by a reputable company and the model is well tested in the field. Prior to deployment, all collars will be rigorously tested to ensure working condition.

All functions of the collars will be utilised, including the built-in accelerometer that indicates activity levels and head position, and the VHF beacon for relocations in the field. The GPS data, themselves, will provide multiple data analysis options to answer the research questions. For example, GPS locations can provide information on temporal distributions, in addition to a subset of the locations being used to determine habitat selection. GPS and activity data can also be combined to look at activity in response to specific events or conditions. Taken together, these features of the collars mean that we will need to collar fewer animals to gather large quantities of data to provide a robust answer to our research questions. That, in turn, means that we are confident of gathering informative data, even with a maximum of 30 adults (a modest sample size for a GPS collaring study that considers a range of determinants of movement in animals; https://doi.org/10.1098/rstb.2010.0087).

We have noted that we will tag up to 30 calves. The more calves we can tag, the more they will supplement our understanding of the movements of relatively hard-to-capture adult females. In practice, experience shows that calf tagging will be constrained by the extreme difficulty of finding well concealed calves in extensive terrain. During previous work with a single field worker, we were only able to find 18 calves over 2 breeding seasons of intensive searching; 30 is, thus, very much a maximum ambition.

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 alongside local stalkers, gaining from their knowledge to ensure that we target animals that maximise the scope of data collection (choosing animals from different herds and at different demographic stages), to ensure that we rapidly gain insights into movements representative of the wider population. Additional measures include the use of data from related studies, that allow us to run computer simulations to bolster our confidence that the number of animals used will be adequate.

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.

Red deer have been chosen for this study because they are the only relevant species in the context of the research question, with alternatives such as domestic ungulates or farmed deer unsuitable. Red deer, and other wild deer species, are commonly and successfully used in similar research projects involving capture and fitting GPS collars.

Methods for capture have been developed with veterinary advice to ensure they are the most refined for the purpose, including anaesthesia with recommended drug doses and combinations. A number of further steps (see further, below) will be taken to minimise animal suffering and stress during the capture procedure and immediately thereafter, and for the duration of collar deployment.

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

The focus of this study is on the red deer, which is the specific organism of ecological, cultural and economic interest in this context. The research questions and context would not apply to another species.

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

The protocol will be conducted only by vets or by other suitably qualified personnel who have satisfied the vets of their competency under supervision, as agreed with the NTCO.

Darting will not occur during times when deer may experience long-term impacts of capture. Specifically, we will avoid darting until 4-6 weeks after the end of the rut (mating) period (to avoid disrupting mating or early pregnancy) and we will not dart during late-stage pregnancy, or when offspring are heavily dependent on their mothers (hence, we will avoid the period May to August).

The primary method of adult capture is to use a dart gun to administer the anaesthetic to an unconfined animal. Veterinary advice is that, to ensure that enough animals can be captured, it is possible that clover traps or corrals could be used to confine the deer for a short amount of time. If confinement is deemed necessary, it will be limited to 12 hours, during which time the deer will not suffer from dehydration and appropriate feed will be available. If remote darting is not practical, anaesthetic will be administered intramuscularly to an animal once safely restrained.

We will only conduct darting where there are no features in the terrain that may cause injury on induction and recovery. Failure to rejoin the herd is not a documented problem but, to avoid concerns, we will work only with fully independent adults. Using an expert and competent team for darting and collaring ensures that the time away from the herd will be kept to a minimum.

During the capture, every effort will be made to minimise stress to the deer. The deer will first be approached from behind whilst watching for eye, ear and head movements that can signal consciousness. First contact with the deer will be a light touch to the hind quarters so that the response can be safely observed, and a blindfold will be put on over the deer’s eyes to minimise external stimuli. To reduce likelihood of injury on releasing the deer, all persons will move away from the deer, allowing the deer an obvious escape route, clear from obstacles, and in the direction the deer is facing.

Monitoring of adults will be carried out remotely via transmitted GPS locations or using the VHF tracking beacon. Collars are purpose-designed to minimise impact on the animal (weight within recommended parameters and appropriate belt-thickness). Collars will feature drop-off devices and will be retrieved using a VHF tracking beacon for recovery of data and to prevent environmental contamination. Devices that allow collars to drop off without the need for recapture are commonly used in studies of this nature. Data from mountainous regions of Central Europe reveal a 98% recovery rate for collars that have dropped off Alpine chamois and ibex. In some studies of primates, individuals have worn collars for up to 6 years without evident problems (Klegarth et al. 2019 in "GPS and GIS for primatologists: applications of spatial analysis in primate behavioral ecology". Cambridge University Press). The use of drop-off mechanisms on the collars ensures that the collars can be collected using the VHF beacon, without the need for recapture of the study animals. Collars also feature mortality signals, notifying researchers of the final location of the animal if it dies before the collar is removed.

Red deer calves will be located from a distance and approached only when the adult female has moved away. A staging ground for preparing tags will be located no less than 15 m from the calf to minimise the impact of human scent in the immediate vicinity of the calf, reducing the risk of rejection or delays in the return of the mother. Once approached, calves will require minimal restraint, due to the freeze response observed among deer calves within a few days of their birth.

The risks associated with tagging calves are 1) visual impact or scent transfer resulting in rejection by mother, or 2) the tag becoming caught in fences or branches. An additional possibility is that the weight of the tracker affects the development of the cartilage in the ear, though this does not affect the function of the ear. The visual impact of the tag will be minimised by painting it brown with a watersoluble paint, and orienting it (initially) on the ear to minimise the impact on the overall outline of the ear. Handling of the calf will be carried out wearing medical gloves rubbed with moss and vegetation. This helps minimise the transfer of human scent onto the calf, reducing the risk of rejection by the mother. The tag will be positioned low on the ear, which reduces the chance of it being caught on fences or branches. Positioning of the tag nearer to the base of the ear also helps prevent any impact on the development of the ear. The tags are lightweight, weighing up to 20g, and are well within the recommended weight limits of tracking devices (1% of total body weight).

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

There is no published best practice for this type of work but two leading studies are those of the red deer on Rum (http://rumdeer.biology.ed.ac.uk; e.g., https://doi.org/10.1006/anbe.2003.2078) and the elk in Ya Ha Tinda (http://www.umt.edu/yahatinda/; e.g., https://doi.org/10.1111/oik.05304). One of the field team for the project we are proposing here has worked on both of these studies, and we have incorporated best practice from both by reference to their field protocols.

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

For more general issues around the 3Rs, we will keep up to date via peer-reviewed journals and contacts within relevant agencies, with which we have active collaborations. For information specifically relevant to deer, we are part of a small, informal network of deer researchers in the UK and we have communicated across this network regarding project design - both for our own and others' projects.

23. Neurophysiological Control of Reproduction

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 animalsreared for agricultural purposes

Key words

Reproduction, Neuroendocrinology, Neurophysiology, Neuronal Manipulation, Infertility

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?

Studying how the brain and nervous system control the body's reproductive system during pubertal development (the stage when people start developing sexually) and throughout the menstrual cycle. The goal is to understand how these processes work and how problems with these processes might lead to infertility, or difficulty getting pregnant.

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?

Infertility is a significant problem that affects animal and human health. Learning more about how the brain and hormones control reproduction and the causes of infertility can have big benefits. It could improve people's health and well-being in society, and also help with creating better medical treatments for infertility. Additionally, this knowledge could be

used in farming to improve breeding practices, leading to better animal health, higher production, and more sustainable farming.

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

This research is focused on better understanding how the brain controls the reproductive system, particularly the hypothalamic-pituitary-gonadal (HPG) axis, which is responsible for regulating reproduction. A key part of this system is the pulse generator in the hypothalamus (in the brain), which controls the release of hormones in a pulsatile fashion that drive the reproductive process. The study will explore how this pulse generator works and how other factors, like hormones and signals from the brain, regulate it.

The research will also look at how this system functions in both healthy individuals and in those with reproductive issues, such as infertility. The goal is to uncover new insights that could lead to better treatments for reproductive problems. These findings will be shared in academic journals, helping other researchers and healthcare professionals better understand and treat conditions related to reproduction.

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

The main immediate benefit of this research is to increase our understanding of how the brain controls reproduction, specifically expanding our knowledge about how the brain governs reproduction via the

"GnRH pulse generator" a key player in the reproductive process. This includes studying how reproduction works in normal conditions and what happens in cases of infertility or other reproductive issues. The findings will be shared in academic journals, targeting scientists who specialize in reproductive physiology, neuroendocrinology, or stress-related research.

In the long term, there are potential clinical applications for this research. For example, understanding how the hypothalamic-pituitary-gonadal axis works under stress could help develop new treatments for reproductive problems, like ovulatory dysfunction, or for stress-related conditions such as anxiety and depression. This could also lead to better management of symptoms like hot flushes in cancer patients or postmenopausal women.

Additionally, the research could have benefits beyond human health. It may offer insights into improving animal breeding practices and welfare, helping farmers develop better strategies for livestock productivity and reproductive health. Ultimately, this research could lead to new drugs and approaches to treat a variety of reproductive and stress-related disorders.

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

Our methods and results will be published in a variety of journals including open-access journals so that this work reaches more scientists and the general public. Results will also be presented at scientific meetings as well as national and international conferences. We have collaborations with researchers from other institutions, including internationally so this work reaches other fields as well as our own.

Species and numbers of animals expected to be used

- Mice: 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.

Rodents such as mice, are a key species used in reproductive research because they are the smallest vertebrates that allow for reliable studies of brain function related to reproduction. Their reproductive control systems are well understood, making them ideal for studying how the brain regulates reproduction. Researchers can monitor brain activity associated with reproduction using techniques like mini-endoscopes (a tiny flexible tube with a camera) with special lenses to track neural activity or by taking blood samples to measure hormone levels like luteinizing hormone (LH), which plays a key role in reproduction.

Since the study also explores how early life stress affects reproductive function, both juvenile (young) and adult animals will be used. The research also involves pregnant animals, as the lab follows its own breeding protocol for these studies.

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

Each animal in the study can potentially be given up to five general anaesthetics, but usually, they only receive a maximum of two, because most of the procedures require just up to two surgeries.

In the typical procedure, very small amounts of light-sensitive proteins are injected into the brain. After that, a tiny fiber-optic probe is implanted, which allows for study of brain activity using light.

Alternatively, a small tube is inserted into the brain to deliver drugs directly to specific areas. These drugs are not expected to cause any harmful effects. In some cases, instead of the cannula (a small tube), a special lens called a GRadient-INdex (GRIN) lens is implanted into the brain with an attachable mini-camera to allow for real-time imaging of brain activity. These procedures are often done together with the removal of the animal's reproductive organs. After the surgeries, the animals may have blood samples taken for hormone analysis.

In about 25% of cases, some animals will be exposed to controlled environmental stress (like changes in temperature or noise) to study how stress affects the reproductive system. This stress is not expected to cause long-term harm, but the animals will be carefully monitored, and extra care will be taken if the stress levels go beyond what is planned.

The studies typically last 6 to 12 weeks, but they may be extended up to 12 months if needed.

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

Surgery can cause pain which is expected. Any animal that undergoes surgery will receive pain relief before the procedure, and more will be given afterward if needed. Each animal will be carefully watched after surgery to make sure they are not in pain, and they will get more pain relief if necessary. If, after 48 hours, an animal has not returned to acting normal—such as not moving well, not grooming itself, or showing signs like a hunched back, raised fur, or losing more weight compared to healthy animals—the veterinary surgeon will be contacted to check on its condition. If the animal’s condition doesn’t improve, it may be humanely euthanized to prevent suffering.

It is rare for animals to have problems while under general anaesthesia (typically 2 hours), but it's possible for the anaesthesia to become too light. This can be checked by testing their response to a pinch or the eye blink reflex. If this happens, more anaesthesia will be given. Animals may also experience a drop in body temperature during anaesthesia, so they will be placed in a heated area or on a heating mat to help them warm up as they recover. The animals will be carefully monitored to make sure they are fully awake and stable before being returned to their cage. Some animals may die due to complications from the anaesthesia- it is important to be aware of this very rare but serious complication.

After surgery, closing the skin can sometimes cause local pain or tissue damage. To reduce this risk, any stitches or clips that don't dissolve will be removed within 7 to 10 days after the surgery. Although it's rare, if a wound reopens, it will be treated by carefully closing it again under short-term anaesthesia, but this will only happen within the first 48 hours after surgery.

Early life stress can sometimes have negative effects. When handling newborn animals, there is a very small chance that the mother might harm or even eat her young, although this is extremely rare.

Gloves will be worn when handling the newborns to reduce the chance of this happening, and the smell of the home cage will be rubbed on them before they are returned to the mother. As the animals grow up, they will be closely watched for any signs of health problems. If any animals show signs of illness, they will be monitored carefully, and if needed, they will be humanely euthanized. It's important to note that post-weaning (after they are separated from their mother) social isolation is not expected to cause any health or growth problems in the animals

Possible negative effects of other stressors on adult animals:-

Short term restraint stress: Holding an animal in a restraining device can sometimes cause distress, such as struggling, which could lead to injury. Animals will be closely watched while restrained, and if they show signs of distress, they will be removed from the device immediately

Acute immune challenge: When animals are given a small dose of lipopolysaccharide (a substance from bacteria like E. coli), they may experience fever which is expected to pass quickly. They may also show loss of appetite, and weight loss. If these symptoms last for more than 12 hours, the animal will be humanely euthanized. However, because the doses of lipopolysaccharide used are very small, these symptoms usually only last for 1-3 hours and are not expected to cause long-term harm.

For other types of stress, such as insulin-induced low blood sugar, noise, predator smells, higher temperatures, or switching cages, no negative effects are expected, and none have been observed.

One possible side effect of adrenalectomy (surgery to remove the adrenal glands) is an imbalance in electrolytes (important minerals/salts in the body). This risk is reduced by adding extra salt to the animals' drinking water. No other negative effects are expected from the procedure- the missing adrenal hormones will also be replaced in the drinking water

Adverse reactions to drug administration (such as through the brain or intravenously through veins) are very rare. If any serious reaction does occur, the animal will be humanely euthanized.

To prevent any infection at injection sites, needles will be changed per animal and injections sites will be switched around for e.g. between left and right side per injection. If any infection is found it will be monitored and checked by the Named Veterinary Surgeon.

A possible side effect of implanting devices like catheters, pumps, transmitters, brain fluid cannulas, fiber optic probes, GRadient-INdex (GRIN) lenses for brain imaging, or screws (used to hold implants in place) is infection at the surgery site, although this is very rare. To prevent this, the surgery will be done using sterile techniques. If an infection occurs, the animal will be treated with antibiotics based on the advice of the veterinary surgeon, and the wound will be carefully monitored. If the infection doesn't improve, the experiment will be stopped, and the animal will be humanely euthanized. Wound breakdown after surgery is very uncommon, but if it happens, the wound will be re-closed under anaesthesia, within 48 hours of the first operation, on one occasion only.

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 animals will undergo surgery (moderate severity) and around 25% may be exposed to stressful environments (mild to moderate severity).

After recovery from surgery, approximately 20% of animals will have blood taken to monitor reproduction related hormones in the blood (such as luteinising hormone. About 60% of animals will also have a special micro-lens called a GRadient-INdex (GRIN) lens implanted, with an attachable mini-camera. This camera allows visual monitoring of calcium events in the brain. These events act as a proxy for brain activity in specific areas which allows researchers to track the functioning of the GnRH pulse generator, an area in the brain that regulates reproduction.

Because this pulse generator is highly sensitive to stress, the animals will be carefully handled and given time to become accustomed to their environment. This is essential for accurate studying of the normal pattern of hormone pulses without interference from the stress of their environment.

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?

This research will use both laboratory techniques and animal models to study how the brain controls reproduction. The in-vitro (lab-based) part of the work will involve looking at cells and tissues to understand how signals are transmitted within cells and how genes are activated. This will help us learn about the pathways and mechanisms that control reproduction at a molecular level.

However, studying the "GnRH pulse generator", which is crucial for reproduction, requires working with animals. This is because the GnRH pulse generator operates as part of a

complex, integrated system of the body, and its function cannot be fully understood in isolated cells alone. For example, to study how stress or changes in diet (like food withdrawal or restraint) affect reproduction, animal models are needed. These stressors are relevant to human conditions like anxiety and anorexia, so using animals allows for a more accurate representation of these factors in real-life situations.

By combining both in vitro studies (cellular research) and in vivo studies (animal models), the research will provide a more comprehensive understanding. For example, examining how stress influences gene expression or neuropeptide activity in animals can be enriched by understanding the molecular mechanisms in cells.

In some cases, the research will involve brain slices from animals, which will be taken humanely using approved methods. These slices will be used to study specific events, like how certain molecules (such as GABA, glutamate, or kisspeptin) are expressed during puberty, giving further insight into the brain's control of reproduction.

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

In addition to using animal models, the research will also make use of non-animal methods. One key approach is studying biological activity in cell lines and tissues. By using molecular biology techniques, researchers can explore how signals are transmitted within cells and how genes are turned on or off, helping to understand the basic mechanisms that control reproduction.

Another important part of the research is the use of computational modelling. This approach involves using computer simulations to model how the GnRH pulse generator works in the brain, and how it is influenced by factors like stress. The in-silico (computational) models are extremely useful tools because they can predict the outcomes of various experiments. This reduces the need for some animal studies, making the research more efficient and ethical as less animals are required. In fact, the predictive power of these models can even replace certain experiments altogether, further reducing reliance on animals.

So, while animal research is necessary for understanding the complex, integrated systems that regulate reproduction, these non-animal techniques help streamline the process and minimize the number of animals required, making the overall research more ethical and impactful.

Why were they not suitable?

In summary, while non-animal techniques are valuable and reduce the need for animal testing, some aspects of this research—particularly those involving complex physiological interactions and specific events in the reproductive cycle, especially those that work as part of a bigger, more integrated system still require animal models for a complete understanding.

Using cells alone is not the same as studying a whole animal under stress. Additionally there are events in the reproductive cycle that are too complex to study in isolated cells and require tissue from living animals to observe them in the context of an intact, functioning system.

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 valuable previous experience making sure we use as few animals as possible in our research. Before starting experiments, we use standard statistical methods that help us determine the the minimum number of animals needed to get accurate results. This helps us avoid using more animals than necessary while still making sure the results are reliable. It's all about being efficient and ethical in how we conduct our research.

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

We always make sure that we conduct statistically and scientifically rigorous experiments. The design of each experiment is discussed by at least two people (myself and the team member who will carry out the work). However, frequently, we additionally discuss particular designs as a group in internal and external lab meetings (e.g. with mathematical collaborators).

My staff are trained in the use of design frameworks, e.g. right now we follow the recently published EQIPD framework (https://www.nature.com/articles/s41592-022-01615-y), and of course always use ARRIVE 2.0 guidelines for reporting.

For example, we use randomisation and blinding, and statistical methods to ensure that we use the right number of animals for each question at hand. We plan within-animal designs where possible to increase our statistical rigor, and we carefully think about which statistical test will give us the maximum information for a given dataset.

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

When animals are used, we will make sure to use the tissue from those animals as much as possible. For example, tissue will be analysed histologically to study specific events like the expression of important molecules (e.g., GABA, glutamate, kisspeptin) during puberty, or how stress-related proteins in the brain are expressed. This approach maximizes the data we can gather from each animal, ensuring that as few animals as possible are used throughout the research.

Additionally, we'll use computer-based (in-silico) models to simulate how different neuroendocrine pathways interact. This approach helps us predict results, reducing the need for more animals in future experiments and can actually remove the need for certain experiments altogether.

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 mice to study:

How the brain controls reproduction. A neural system in the brain that causes the pulsatile release of hormones necessary for fertility is poorly understood. Monitoring the activity of these neurones using miniature lenses and cameras or electrodes developed and refined for the mouse is essential to our understanding of reproductive biology.

We now have the capacity using sophisticated viral constructs to selectively alter the activity of neurones and their neurotransmitter composition and this unprecedented level of refinement means that we can discover at the molecular level the function of the individual neurones that control reproduction. This level of detail is critical to our understanding on reproduction.

To understand how stress cause infertility and other reproductive disorders, we have developed stress modalities, for example insulin-induced hypoglycaemia, that replicate metabolic stress, and monitor changes the brain controlling reproductive function. All surgical procedures are performed under appropriate anaesthesia with analgesia. During and after all procedures mice will be carefully monitored. In our experience, mice do not show signs of pain or suffering, eating and drinking normally.

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

Rodents, like mice and rats, are the best animals for studying how the brain controls reproduction because their reproductive systems are well understood and they can be used for accurate brain activity studies.

Less sentient animals such as drosophila and zebrafish either lack a GnRH pulse generator or do not have one that is as well characterised compared to mammalian species and would therefore not be suitable in terms of the translation/application of results.

To study the GnRH pulse generator, we use juvenile or adult animals. This is because the control of reproduction involves many different body systems working together, which can only be fully studied in more mature animals as opposed to in simpler or younger models. Using juvenile or adult rodents gives us a complete picture of how the system works in a fully developed organism.

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

Animals will be closely monitored throughout all experiments to ensure they experience as little pain or discomfort as possible. If any animal is found to be suffering, it will be humanely euthanized using an approved method (Schedule 1).

Before any surgery, pain relief (analgesia) will be given to all animals, and animals will not be used in experiments if it shows signs of pain or distress. Most of the surgical procedures and treatments we plan to use have minimal, if any, lasting negative effects.

In rare cases, certain procedures, such as those involving brain surgery, could cause side effects, such as reduced mobility or appetite. If this happens and the animal shows signs of poor health, such as weight loss, a rough coat, or unusual behaviour, the named vet will

be consulted and it will be immediately and humanely euthanized to prevent further suffering

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

We will follow the National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs) and the ARRIVE guidelines as well as ensuring we keep up to date on practices in the current literature 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 up to date on new advances in the 3Rs through the NC3Rs newsletter as well as regularly checking the 3Rs website.

24. Drug Metabolism and Pharmacokinetics

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

Pharmacokinetics, Absorption, Distribution, Metabolism, Excretion

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?

A key step to select the most promising substances for further development as medicines for serious human and animal diseases is to generate information on what the body does to a substance over a period of time (pharmacokinetics) and how new substances are absorbed, where they go in the body, how long they remain in the body, how they are broken down and how the body eliminates them (known as Absorption, Distribution, Metabolism and Excretion studies or ADME). This will inform our decision making on whether a substance should progress within a drug discovery and development programme for any disease area, and direct what changes to a substance a medicinal chemist may need to do to further improve it for therapeutic benefit. This project will not support investigations on household products or cosmetics.

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 studies to be performed under this project shall support a number of drug discovery programmes within the establishment. The specific areas being investigated by these

programmes are wideranging, however a majority of the current programmes focus on the development of treatments for infectious diseases with significant global health burden, for example, tuberculosis, malaria and Chagas' disease. Tuberculosis is a bacterial infection recognised by the World Health Organisation as being the world's top infectious killer and a major contributor to antimicrobial resistance, with the disease infecting around 10 million people per year and killing around 1.5 million. Malaria is a parasitic infection estimated to have caused 249 million cases of infection in 2022 alone, including around 608,000 deaths. Chagas disease, caused by infection with kinetoplastid parasites (these are organisms that are made of a single cell), leads to around 15,000 deaths per year, and an estimated 7 million people are thought to be infected. Other drug discovery programmes also supported by this project include anti-viral treatments, and non-hormonal contraceptives.

Pharmacokinetic studies are integral to any drug discovery and development programme, and requires the use of animals as there are currently no suitable non-animal methods available. Pharmacokinetic studies are usually small-scale studies which measure the movement of a substance throughout the body. The data gained is used to inform decision-making processes, provide insight to allow medicinal chemists to create further improvements for a substance to be active in the body, and remove unsuitable substances from the drug-discovery pipeline while using very few animals.

The information gained at this early stage is also useful to inform later drug development for successful candidate substances, including the best way of performing the legally required safety testing , and how to work out relevant dose levels for human patients.

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

The pharmacokinetic data generated in this project, such as substance concentration with time or calculated key pharmacokinetic parameters (clearance, volume of distribution, half life, bioavailability etc) derived from the concentration with time curve, will be essential for decision making in drug discovery and development programmes which seek to discover better and safer treatments for human and animal disease.

We expect our findings to be published in research articles focused on the areas of disease being targeted by each drug discovery programme.

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

In the short term, this work will provide essential information on how potential new treatments act in a whole body. This information will be used to help design later studies to prove these treatments deliver the desired benefit in a whole body and are safe to take, or to make further improvements to those treatments.

In the medium term, potential treatments with the best results would be moved forward through the "drug development" stage, where they are tested in regulatory studies, eventually including clinical trials (research studies on human volunteers and then patients). To increase the likelihood of a treatment being successful throughout the drug development stage and reaching the patient population, each drug discovery programme aims to have multiple potential treatments reach this stage.

The long-term benefit would be the delivery of new and safe drug treatments, that will have a major impact on the health and wellbeing of people. This shall benefit healthcare providers by overcoming the problems associated with currently available treatments and improving patient compliance. Patients shall benefit from improved outcomes of treatment.

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

This work is conducted in the context of an integrated drug discovery programme, where different types of specialised scientists from the establishment and our collaborators work together to produce new potential treatments. Where possible, we will share data, substances we make or use, and our methods with other researchers in the disease areas we study, and with other drug discovery programmes which may also benefit from the information. Where possible, datasets and publications will be open access so that they can be easily accessed by other researchers and members of the public. When we publish articles, all methods shall be described in detail. The information we gain, and our expertise in the interpretation of this data, shall be used by drug discovery programmes to guide the successful development of new treatments.

Species and numbers of animals expected to be used

- Mice: 4050
- Rats: 675

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 majority of drug discovery programmes we work with primarily use mice, as they are the lowest animals which are relevant to the disease areas being studied. For example, many of the infectious diseases being studied are not capable of infecting insects or fish, so must be tested in mammals such as mice. Mice are also available with informative genetic alterations, some of which are essential to allow human treatments for certain diseases to be designed and tested.

Rats may be used where drug metabolism studies indicate that mice are unsuitable for certain substances; where the disease model in rats is more relevant to the human disease than mice; or where pharmacokinetic data from rats is needed to develop mathematical models which are needed to enable prediction of the whole body performance of a drug from its cell-based results, and eventual prediction of dose requirements in humans. Rats are also the preferred rodent species for later toxicology testing, as toxicology testing usually requires larger blood samples to test a much larger range of potential changes in the blood. These blood samples can be collected from rats noninvasively and with minimal harms. Samples of this size generally cannot be collected from mice without using methods under terminal anaesthesia. We therefore need to ensure our advanced lead substances have good rat pharmacokinetics to be able to smoothly proceed through the later stages of preclinical drug development.

Genetically modified or spontaneous mutant animals may be used where genetically normal “wild-type” animals are not suitable to provide the needed scientific outcome. For example, mice which are “humanised” (where mouse genes have been replaced with human genes) for drug metabolism may be used to improve the relevance to human patients if cell-based studies find differences in how a treatment is metabolised by human cells and normal mouse cells. Alternatively, if larger-scale studies to investigate the effectiveness of a potential treatment requires the use of animals with a genetic alteration relevant to a particular disease, the pharmacokinetic studies performed under this project would use the same animal strain, to ensure that the resulting data is relevant to the

selection of substances for those larger-scale studies, and thereby reduce unnecessary animal usage.

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

For studies under Protocol 1 of this licence, the majority of animals shall receive a single injection or oral dose of an experimental substance, followed by small blood samples being collected from a peripheral vein (for example, the tail) at various time points, typically up to 8- or 24-hours after administration. A subset of studies will have a longer duration (usually 5 days), either where compounds being investigated are expected to last for a very long time in the body, or for dose-ranging studies (where multiple increasing doses of the same compound are given to evaluate whether factors such as absorption or metabolism change as the dose amount increases). Our animals are usually kept in groups, but occasionally some animals have to be put on their own in a specialised type of cage, with a grid floor, that lets us collect urine and faeces over a short period of time (usually no more than 36 hours), in order to provide useful information on the elimination of the substance under investigation from the body.

For studies under Protocol 2, the majority of animals are placed under terminal anaesthesia for the collection of blood prior to being humanely killed. Tissues are also usually collected to ensure maximum use of each animal.

Under protocol 3, a small number of animals may receive a single administration of a safe, non-toxic vehicle (a vehicle is a substance, or mixture, in which an experimental substance could be mixed with to allow it to be administered) prior to being placed under terminal anaesthesia. This would only be used where it is suspected that a vehicle used in Protocol 1 or 2 may have an effect on the normal blood profile of an animal.

Protocols 4 is for the purpose of maintaining genetically altered mice before they move on to studies that are run on the other protocols of this licence.

Protocol 5 is for the purpose of providing information on the movement of a substance within a whole body at steady-state.

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

In these studies, animals may have transient discomfort associated with the administration of experimental substances when given through an injection or by gavage (when given by a tube which is passed down the throat into the stomach). Adverse effects caused by the administration of substances are not generally expected as experimental substances will already have been tested via cell-based systems to rule out various forms of toxicity. However, there is always a risk, especially when testing a substance for the first time in a whole animal, that the substance may cause unexpected adverse effects that was not predicted due to the limitations of previous cell-based systems. Additionally, there is a risk on studies longer than 24 hours (for example, to investigate substances which last a long time in the body, or for studies where increasing amounts of a substance are tested) that longer-term adverse effects such as weight loss may become apparent, which would not have been seen in shorter studies.

Blood sampling via the tail vein is not expected to cause adverse effects beyond the transient discomfort of the blood sampling itself, as the sampling volumes are low and the total volumes collected are always within the limit of 15% of the total blood volume in 28 days which is recommended by the National Centre for the 3Rs (NC3Rs).

Mice may be genetically altered. Mice may be used with a range of genetic changes, but we often use mice where the enzymes that are involved in how drugs are handled by the body are changed. The majority of genetic alterations are not expected to affect animal welfare. However, one of the lines where mouse metabolic enzymes have been “swapped” for human ones (needed to better mimic what happens in people for some types of drugs) sometimes show some adverse clinical signs (specifically reduced responsiveness, and a fall in body temperature). This happens in around 10% of animals from this line. These animals have to be humanely killed to prevent them from becoming very seriously unwell. The occurrence of these adverse clinical signs is currently thought to be related to stressors such as a change in diet. Animals who do not show these signs remain well, do not have a risk of being less well than normal (“wild type”) animals in studies and produce extremely valuable and reliable results. We are currently looking at ways that we can stop the risk of this line of animals having these 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)?

We expect the majority of animals to experience a mild severity, though a small percentage of animals may experience moderate severity due to unexpected adverse effects related to the substance being tested having some side effects (for example causing weight loss or sleepiness). During our previous licence, we observed that approximately 3% of all animals experienced a moderate actual severity, while 64% of all animals experienced a mild severity.

Approximately 26% of all animals under the previous licence experienced an actual severity of nonrecovery, where the animal received no intervention prior to being placed under terminal anaesthesia.

Under our previous licence, approximately 7% of animals experienced an actual severity of below threshold (so had less pain / discomfort than you would get from a single injection). The majority of these instances were related to the breeding of genetically altered animals conducted under out previous licence, which is not required under this licence, so we expect this percentage to be lower for this licence. A small number of those consisted of genetically altered animals which received no intervention at all but were humanely killed by an approved method to provide tissues. This may happen during this licence.

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?

While they are not sufficient to fully predict the pharmacokinetics and tolerability of novel potential drug treatments, non-animal methods are used in order to initially develop and improve substances prior animal work being carried out under this project. Cell-based systems are used to find substances which appear to be effective, determine how they

work, select substances which have appropriate characteristics for use in animals , as well as humans, (such as how well they will dissolve inside the intestines, and how quickly they are metabolised), and to screen for various forms of toxicity. Cellbased tests are carried out using cells from humans, and from the species of animals which are likely to be used in efficacy and later toxicology testing. Computer modelling is also used to help improve substance design, to predict the required doses needed to be effective in animals, and to predict potential issues with toxicity or “off-target” activity (the term for when a drug affects something else in addition to what it was designed for).

Animals need to be used because there are currently no reliable alternatives currently available to completely replace pharmacokinetic studies in a whole body for a new series of treatment substances. The information generated from these small-scale studies are essential to create better treatments, and inform further follow up studies where animals are required, in a way which minimises the risk and overall number of animals used.

The analysis of samples from animals on pharmacokinetic studies requires a supply of control blood and control tissues from the same species, strain, and sex of animal as is used on the pharmacokinetic study itself. This is because small changes in response to a control solution could have substantial impact on the results from experimental animals and progression of potential treatments. Some laboratory tests also require blood portions or tissues from animals, such as plasma to measure the amount of unbound substance in the blood (which is used to determine how much substance is available to engage with the desired target) or liver cells to determine the rate of metabolism.

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

The culture of cells allows for cells to be grown in cell growth solution without the requirement to use fresh or frozen tissues from animals. This was considered as a potential replacement for control tissue animals.

Organ-on-a-chip technology is a cell culture system which uses microfluidic channels and multiple cell types to more closely simulate the cellular environment which would be present in a live animal. The microfluidic channels can be used to deliver culture fluid, or an experimental substance to those cells. Multi-organ-chips are a similar system where multiple organ-on-a-chip systems are connected to a single fluid reservoir, similar to how organs in the body are connected by the bloodstream. These systems were considered as a potential replacement for certain pharmacokinetic studies (e.g. tissue distribution studies).

In silico pharmacokinetic modelling uses computer-based systems to predict the pharmacokinetics of a substance in a live animal at different dose levels, or in different species. Machine-learning systems are a type of computer-based (in silico) artificial intelligence, which uses statistical algorithms to analyse existing data from previously studied substances and use that data to make predictions about other substances. These in silico models were considered for their potential to replace pharmacokinetic studies in animals.

Why were they not suitable?

While some cells can be easily cultured, for others it is not possible to do so in a way in which the response of those cells to a substance sufficiently reflects that seen in a live animal. This is because cultured cells, or mixtures of cells, can differ substantially from the complexity of systems in a whole body. Use of cultured cells for studies in these cases could potentially result in the provision of inaccurate information for decision-making and the removal of otherwise promising treatments or the progression of unsuitable ones.

Additionally, while some cells are easily cultured, it may not be possible to culture them indefinitely so replacement tissues are routinely required to refresh culture stocks, and for some of the animal strains we use, the cells or tissues required are not commercially available so these must be collected and prepared in-house. It is not possible to use cultured cells to replicate the controls needed for analysis of biological samples, as small changes in a cell system in comparison to live animals on study can have substantial impact on the accuracy of results and could compromise both the progression of promising treatments and the good design of follow up studies.

Organ-on-a-chip technology shows promise to improve the testing of compounds prior to progression for use in animals, however there are issues in culturing microenvironments on these chips which do not accurately reflect certain single-organ systems in the live animal, notably in modelling factors such as the blood brain barrier which is key in limiting the ability of a large number of substances to enter the brain and is an essential factor for some drug discovery programmes. Additionally, they require cells which can be easily grown in culture, which may not be possible with all tissue types. However, significant progress has been made in more recent years to improve these systems, so they are likely to become more useful in investigating metabolism in target organs as the current issues are overcome, and potentially reduce animal numbers in the future. Unfortunately, their use as a complete replacement for whole-body pharmacokinetic studies is currently limited by the number of tissues which can be tested in a microfluidic system. Absorption, Distribution, Metabolism and Excretion (ADME) are processes which involve multiple organs and complex processes between those organs which impact overall drug exposure in each tissue. Multi-Organ chips which are currently described in the literature face significant challenges with limited types or numbers of tissues, the replication of fully functional cellular barriers mimicking the blood vessels, as well as the selection of culture solutions which are suitable for all of the tissues being cultured.

In silico (computer-based) modelling of pharmacokinetics has potential to improve the selection and design of substances in known chemical series, and to improve later-stage study design where some data from live animals exists. Computer modelling systems based around this are already being used by our group and collaborators to predict appropriate dose levels, especially where some pharmacokinetic information is already available. However, these systems are relatively poor at predicting the pharmacokinetics of substances which are completely untested in live animals, especially when those substances are from a new chemical series with limited existing information. This is in part due to the limitations in attempting to predict unexpected effects of new substances in every tissue type in the body using cell based test results. Our group has experienced multiple cases under our previous licence period where experimental substances displayed poor correlation between the prior computer modelling and the results obtained in animals. Most drug discovery programmes consist of a significant portion of new chemical entities from previously untested chemical series, and data from animal studies are required to build and maintain accurate models to assist with decisionmaking. Until prediction of pharmacokinetics for new substances can be improved, in silico pharmacokinetic modelling is unlikely to be able to completely replace animal studies.

Machine-learning systems face similar challenges, as a significant amount of data is required to build and improve the accuracy of these systems for each new chemical series. Some machine-learning systems are currently being developed in-house using existing data to help remove more unsuitable substances at an earlier stage (for example substances with a chemical structure which is likely to be very rapidly metabolised by the liver), which in turn may reduce the number of animals required over time to support cell based studies. However, this is unlikely to replace animals completely in the near future

due to the amount of data required for each chemical series to reduce the uncertainty and error to an acceptable level.

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?

Pharmacokinetic (PK) studies do not provide quantitative data, and so as few animals as possible are used. Typically, 3 animals are used for each “phase” of a pharmacokinetic study, as the use of only one or two animals carries a risk that variability between individual animals could result in differences in blood concentration and PK parameters which make interpretation of results and subsequent study design difficult. Three animals are considered sufficient to provide the mean and standard deviation for blood concentrations and provide useful data for decision-making and later study design. Where tissues are required to be collected at specific times after dose administration, a single animal can only provide data for one time-point, so larger numbers of animals (typically 9) may be required to form a meaningful result of drug concentration in those tissues over time.

The first pharmacokinetic study for a substance will usually consist of two “phases” - one intravenous phase and one phase by the intended route in humans (usually oral). Any subsequent studies (for example dose escalation studies to identify the optimal dose for levels of active drug) will ordinarily only have a single phase by the intended dose route in humans. Rat studies with two phases (i.e. an intravenous phase and an extravascular/oral phase) may use the same rats for both phases, provided that they have suffered no more than mild and transient effects from the first phase and animals are allowed a period of at least 7 days between the two phases.

The number of studies and phases has been estimated based on: 1) the number of studies typically required to support a single drug discovery programme at various stages of hit discovery and lead optimisation; and 2) the number and stage of the drug discovery programmes which are currently being supported in the establishment and likely to require support in Drug Metabolism and Pharmacokinetics (DMPK) in the short-to-medium term.

For control tissue animals, the number of animals required has been estimated based on the amount of control blood and tissues required for bioanalysis of the previously estimated pharmacokinetic studies, as well as the number of tissues expected to be required to support the in vitro DMPK study requirements of current and upcoming drug discovery programmes.

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

For protocols where multiple blood samples are required, microsampling techniques are used to enable repeat sampling from the same animal and thus obtain a full pharmacokinetic profile from individual animals. This reduces the number of animals required to obtain the data, while also reducing the impact of inter-animal variability on results.

The experimental design of using the same animals on both phases of a 2-phase study (i.e. where both the intravenous route, plus another route such as oral is tested; cross-over design) is already

used at other establishments to reduce animal use in pharmacokinetic studies on larger, non-rodent species, and further reduce inter-animal variability. The smaller volumes of blood collected with microsampling allows for use of the same rats across both phases of a 2-phase study, which reduces the number of animals required with minimal additional harms to the animals used.

Where animals are used for the collection of control tissues, all potentially useful tissues, as well as whole blood or plasma, are collected from each animal to reduce the number of animals required. The blood and various tissues collected from a single animal can support both the analysis of biological samples from multiple pharmacokinetics studies, as well as the tissue requirements of a number of cell based assays.

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

Cell based studies will be used to triage and guide decision making prior to progression to animal studies. The PK data generated in our projects is used to validate the usefulness of cell based clearance (metabolic stability) assays within the same species. Good rodent “in vitro: in vivo correlation” (the term for how well the results of cell-based studies can predict the results of animal studies) within a programme results in these cell based technologies being rigorously applied prior to animal studies being performed, thus improving triaging of compounds for PK studies and leading to a further reduction in animal studies.

Computer modelling will be used where possible to improve dose selection and further reduce 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.

Single-dose pharmacokinetic studies are small-scale studies, which provide important information about the movement of drugs in the body. They are essential to identify the study optimal design and identify the correct dose and dose regimen for subsequent studies, whilst also using as few animals as possible. Animals are administered either single doses, or a few doses as are needed to provide the required data (for example, where multiple doses are needed to reach steady-state blood concentrations). Studies use dose levels which are not generally expected to cause adverse effects.

Serial blood samples (when multiple samples are collected from the same animals) are collected using microsampling techniques, to keep the amount of blood removed from the animal as low as possible. Generally, the collection of these samples involves making a small “nick” or puncture in the vein at the initial sampling, with removal of the scab where possible at subsequent samplings to restart the blood flow. This technique keeps the

number of punctures of the vein as low as possible, and reduces the transient discomfort associated with blood sampling. This also allows blood samples to be collected using very minimal restraint, further reducing any potential stress or discomfort – for example, wellacclimatised rats are often willing to sit unrestrained on the arm of a handler, or another soft surface, while blood samples are being collected.

The collection of tissues and large quantities of blood are always carried out either under terminal anaesthesia, or after the animal has been humanely killed, which prevents the animal from experiencing any pain or suffering from those procedures.

Genetically altered or mutant mice may be used when those mice are also to be used in later studies. Most commonly (e.g. 8HUM mice, humanised for metabolism) this is due to significant differences between mouse and human metabolism, where these differences form barriers to the use of a wildtype mouse model in later studies and there is sufficient evidence of translational improvement when using these genetically altered models. In other cases, the genetically altered strain may be important to the disease area and is to be used in subsequent efficacy studies. In these cases it is important to use the same strain in pharmacokinetic studies for the information obtained to be useful and relevant to inform the design of subsequent studies.

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

Pharmacokinetic studies must use the same species and stage of life as is to be used for later efficacy or proof-of-concept studies within each drug discovery programme, for the information to be useful in decision-making and to reduce the risk of unsuitable treatments being progressed to larger animal studies. The majority of the drug discovery programmes we support primarily use adult mice in these later studies, as they are the lowest animals that are suitable hosts for the diseases being investigated. Programmes may also require the use of rats where mice are not sufficiently representative of the disease being studied. When sufficiently advanced, all programmes will require the use of rats to ensure that rat is suitable for down stream toxicology testing.

Justification for the use of each species is internally assessed for each drug discovery programme being supported.

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

Positive reinforcement training is being used to further decrease any transient stress related to procedures. For example, rats may be trained to be gently held by a handler while an intravenous infusion is given, rather than being placed in a traditional restraining device. If there are occasions where manual restraint is necessary, less restrictive options shall be considered where possible to improve comfort for the animal. For example, the use of soft fabric rather than hard plastic restraint devices.

Options for the use of voluntary consumption of substances in food or treats are being investigated for its potential as a more refined method than oral gavage, though this may not always be possible depending on the solubility, vehicle requirements and taste of the substances being tested.

Furthermore, we will aim to improve options for enrichment in metabolism cages on occasions where their use is scientifically necessary.

The advice of the NACWO/NVS will be sought on matters of animal day to day care and welfare, as required. Animals will be housed in groups where possible and environmental

enrichment provided. Animals will be housed and cared for in conditions that meet or exceed the Code of Practice.

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

LASA good practice guidelines for the administration of substances inform the maximum dosing volumes in mice for each administration route chosen.

Limits on blood withdrawal follow the NC3Rs guidance on blood sampling in mice.

The standard 5-point scale for body condition scoring shall be used alongside absolute bodyweight gain/loss on longer studies to assess the effect of treatments on animals: Ullman-Cullen M and Foltz CJ (1999). Body condition scoring: A rapid and accurate method of assessing health status in mice. Lab. Animal Sci., 49, 319-323.

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

Members of the group receive the e-mail newsletter from the NC3Rs (National Centre for Refinement, reduction, and replacement) as well as information from the local animal users' group about best practices regarding the 3Rs. We attend local training and seek out advice from the Named Veterinary Surgeon (NVS) and the Named Animals Care and Welfare Officer (NACWO).

25. Ageing and regeneration: novel therapeutic opportunities to address biological impacts of ageing and regeneration

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

Ageing, Senescence, Regeneration, Therapeutics, Skin

Animal types	Life stages
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult, Aged animal
Spiny mouse (Acomys Cahirinus, Cairo Spiny mouse)	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?

We aim to explore ageing and regeneration and to use this knowledge to identify new therapeutics to improve the "health-span" in elderly people.

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 UK population is ageing. 12.3 million people were aged 65 or over in 2019. By 2066 this number is estimated to reach 20.4 million (26% of all UK population). We are living longer than at any time in human history, but this victory of public health is accompanied by an unfortunate side-effect: a new epidemic of chronic diseases for which we have no

cure. We want to combat age-related disease by focusing on rejuvenation and regeneration.

This work will study the biology of ageing, to develop new strategies to prevent ageing and promote healing in multiple organs.

At the moment, we focus on the skin as it is an accessible organ that does not regenerate with ageing. This reality of skin ageing has been documented since World War I, with the observation that wounds heal more slowly in older soldiers. A fetus heals wounds without scarring, but a scratch on an elderly person can take months to fully heal. This lack of healing results in infections, dry, itchy skin, etc., that seriously impacts the quality of life of elderly people.

We will also use the spiny mouse (a mouse with high levels of skin regeneration throughout its entire life) to understand how to increase the "health-span" of human skin.

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

Investigations under this license will allow us to identify:

Mechanisms of ageing, rejuvenation and regeneration

The biology that controls skin ageing and regeneration

Novel therapeutic for improving regeneration (in multiple levels, with a particular focus on skin regeneration)

Potential non-interventional analysis and diagnostic technologies for skin ageing and regeneration, allowing a reduction in animal use.

In our group we focus on the biology of ageing, the new plague of the 21st century, with individuals over 65 years old representing over 19% of all UK population. This group is particularly at risk of a number of painful conditions, including skin conditions, with a tremendous impact for the individual and our society, and an approximate cost for the NHS of £8.9M per year.

This line of research is expected to generate publications in peer reviewed journals, patents for novel therapies, presentations at national and international conferences and identification of candidate compounds expected to be progressed to clinical trials.

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

Immediate benefits: scientific and technological advances that will increase the overall knowledge and capabilities of the scientific community.

Medium benefits: by identifying novel pathways, cell populations and signaling mechanisms that control ageing and regeneration we expect to influence these processes (for example in the skin). Our results will facilitate pharmaceutical and cell therapy strategies to influence overall organ regeneration and optimize the healing response during ageing. These pre-clinical strategies have the potential of being rapidly translated into the clinic.

Long term benefits: ultimately, we aim to provide novel therapeutic options for elderly patients. The UK population is ageing rapidly and has limited therapeutic options for age-related conditions. For agerelated skin conditions, we are perfectly positioned to capitalize

on the clinical opportunities offered, as we collaborate with a number of dermatologists and plastic surgeons that will help translate our findings to a clinical setting.

We would also like to highlight that some ageing mechanisms are conserved at cell and tissue level, so any potential results obtained using this license could be applied to other tissues to improve overall ageing.

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

All data produced in the procedures covered under this license will be available via open access, peerreviewed journals.

We expect to deliver a number of journal publications in high-profile journals, conferences and patents that will leverage our aspirations to bring novel technologies to the elderly population.

Species and numbers of animals expected to be used

- Mice: 6500
- Spiny mouse (Acomys Cahirinus, Cairo Spiny mouse): 3500

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: we will use rodents, which are versatile and allow us to mimic clinical conditions. Ageing is a complex process that cannot be fully reproduced using cells in the lab. Mice offer an excellent platform to mimic human ageing. These protocols are designed to be as short as possible to minimize discomfort whilst inducing ageing, that can be assessed using well-validated assays.

Spiny mouse: the spiny mouse is the only mammal that can escape predation by shedding its skin. Interestingly, the spiny can regrow skin, nails and hair with minimal scarring. As we want to understand the process of ageing in the skin (and the lack of regeneration observed with ageing in this organ), we aim to investigate which mechanisms are responsible and apply them to improve human skin regeneration for the elderly population.

For both animal models, we will explore different points of lifespan from birth to late age. We will therefore assess rodents at all developmental stages.

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

A simple experimental procedure could involve **skin wound healing in young and old mice**. To do so, we would do a small incision in the dorsal skin of a young mouse and the same incision in the back of an old mouse and compare the healing process over the course of 14 days (the usual period for a wound to heal).

Another example could be to **administer rejuvenating factors or control substances to an old mice** (e.g., fisetin, an antioxidant found in strawberries that have been shown to delay ageing). We could then assess the cognitive function of those mice as well as assess the biological events in several organs, including skin.

We also aim to **identify factors in the blood and in the skin of the spiny mouse** that help accelerate wound healing. We could then administer those specific blood factors or transplant certain skin cells to induce regeneration in old animals.

Examples of complex procedures in this license include:

Young-to-old skin transplantation: transplantation of skin from a young mouse (donor) into an old immunocompromised mouse (recipient). Donor skin is placed on top of a wound bed in the recipient and sutured. After successful engraftment the transplanted skin could be challenged with a small incision (to test if it still heals). Skin healing can be assessed using a caliper, not requiring anesthesia or mouse restraining.

Paired blood exchange: to test the rejuvenating effects of young blood in an old mouse, we have devised a new procedure that involves connecting the circulatory systems of two mice. Rather than suturing two animals together as its currently done (parabiosis) we will place a permanent catheter in the jugular of the mouse, that is connected to a small button on the nape of the animal. This button can be connected to a freely-moving tube that will allow us to draw or inject specific volumes of blood while the animal is moving free in a normal cage. We believe this system also represents a long-term solution to avoid constant puncture of the animal to draw blood or inject substances directly to the circulatory system, and thus represents a 3R improvement.

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

Expected impacts/adverse effects for each procedure as follows:

Genotype: brief restraint for ear notching is required. In exceptional circumstances (<1%) this may need to be repeated if a technical problem prevents successful results being obtained. Repeated each notching (on one occasion) could cause some discomfort, not requiring analgesia or anaesthesia.

Ageing: mortality rates due to natural death may increase towards 24 months old. Aged mice could develop signs of age (cataracts, increased curvature of the spine, etc) that are clearly different from signs of pain (assessed for example using the grimace scale from NC3Rs. Should the mice present any signs of ill-health (cheek bulge, nose bulge, orbital tightening, reduced social activity, reduced food uptake) they will be promptly humanely culled.

Administration of substances (e.g., drugs and cells): adverse effects will be dependent on animal background, substance of interest and route of administration. Any novel compounds will be initially assessed using a dose-finding procedure (administration of a range of doses to find the lowest, non-toxic, effective dose).

Topical administration can lead to skin irritation and temporary local inflammation. This will be minimized by administering appropriate vehicles to maintain skin moist (e.g., petrolatum jelly).

Systemic administration (e.g., via intraperitoneal, intravenous injection) is not expected to lead to any more than minor and temporary discomfort.

Oral gavage can result in windpipe damage. This will be minimized using appropriate techniques by experienced staff and close monitorization for signs of respiratory distress.

Administration of substances in the diet may cause unpalatability and therefore weight loss which can be reduced by mashing the diet and allowing easy access to hydration for the rodents.

Administration of substances via permanent catheter access or minipump is well tolerated and no adverse effects are expected.

Blood sampling: is not associated with adverse effects. No more than 10% of total blood volume will be extracted at any point .

Irradiation: may damage gut epithelium (resulting in malabsorption of nutrients and weight loss). Damage to the hematopoietic system may cause bone marrow depression (leading to anemia).

Behavior studies: to evaluate balance, grip strength and motor coordination of aged mice. These techniques are generally well-tolerated, but can present with stress, falls, weight loss that will be minimized via continuous supervision, foam padding placed under any movement-related tests and specific number of tests (e.g., no more than 1-2 tests will be performed in the same animal per day).

Surgeries, anesthesia and analgesia: risk of anesthetic death is rare (<1%). Mice temperature and breath will be controlled at all times to minimize risks. Mice will experience some discomfort after surgery and mild to moderate pain that will be treated with analgesics. Potential side effects of the surgeries include infection and delayed healing which will be minimized by use of aseptic techniques, regular post-operative checks and minimization of handling of tissue.

Skin transplantation: can present a low rate (<2%) of graft failure potentially leading to inflammation and infections. Skin grafts will be transplanted in immunocompromised mice to avoid rejection. We use highly efficient suturing techniques and infection will be minimized by regular postoperative checks. Graft detachment will be minimized using sterile gauzes and band-aids to prevent excessive grooming. If detachment occurs, non-infected wounds will be reclosed using appropriate technique following veterinary advice.

Paired blood exchange: will be applied to study the rejuvenating impact of young circulatory factors in old mice. Small volumes of blood can be extracted by placing a catheter in the circulatory system of the mice. Catheter placement requires microsurgery under general anesthesia, and it is well tolerated. The catheter can be accessed via a small button on the nape, not requiring anesthesia or animal restraining.

Skin wound models: skin healing will be induced by superficial wounds. Animals will experience mild discomfort that will be minimized with analgesics. Gauze and band aids could be used to minimize risk of local infections.

Skin cell transplant: cells that induce increased regeneration and/or ageing may be transplanted by placing them on the wounds, inside electrospun scaffolds placed on top of the wound and secured using band-aids or via subcutaneous injection. Animals that receive cells that promote skin ageing may experience a temporary local inflammatory reaction and will be closely monitored and potentially receive analgesics in consultation with the veterinary services.

Immunosuppression: could be used to provide an appropriate frame for cell engraftment, and/or to study the effect of immune cells in our models. A minority of animals may experience post-irradiation immunosuppression and weight loss of less than 10%. These

will be closely monitored and treated with routine prophylactic antibiotics and analgesia in consultation with the veterinary services.

Imaging: may be applied to assess welfare and experimental outputs. We do not expect adverse effects from non-invasive fluorescent imaging or MRI other than those associated with anesthesia.

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% mild, 90% moderate for all animals in this license

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?

Aging is a complex process that cannot be fully summarized by studying individual factors like cells, or using other alternatives (e.g., artificial intelligence models).

Many of the experiments in this project will continue to use human tissue (from biobanks) wherever possible to assess the relevance to human disease. These studies will not only guide the number and experimental design of the animal work but will replace the mouse usage where possible.

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

We have looked at using human tissue slices, in silico data (https://tabula-murissenis.ds.czbiohub.org/), and two/three-dimensional *in vitro* cultures.

We have searched FRAME (http://www.frame.org.uk/) and the website Norecopa (https://norecopa.no/) for suitable *in vitro* alternatives.

Why were they not suitable?

None of these systems accurately explain the process of ageing (as they do not have the complexity of a multicellular response) or only study one interval of a progressive response (e.g., human tissue analysis).

Even though alternative strategies could be designed (such as adding other cell types *in vitro*) the system does not accurately predict how the cells interact at the injured site.

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 estimations from our own earlier experiments) to calculate minimum numbers of animals to be used, whilst ensuring that the results are statistically significant. Calculations typically show that we need group sizes of 8 to achieve the quality of results we need.

Using publicly available data for simulation, sample size calculations were performed with the statistical software Minitab®17.1.0, for detection of a difference between either two means (2-sample t test) or two proportions (test or 2 proportions) with a target power of 0.8 and α=0.05, retrieving a total of 7 and 10 mice respectively. The chose number of mice (approximately 8 per group) is based on the combination of the above sample size calculations and our own experience in pilot experiments performed at a Surgical Skills Centre. Attempted bias reduction would be undertaken by age- and sexmatched, randomised group allocation, as well as by performing analyses blinded to group allocation, where feasible (e.g., imaging analysis).

I have studied the experimental design and data analysis course available online at our Establishment:

biomedical sciences, which helped to design power analysis and understand covariates. We also consulted the Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE) guidelines to ensure quality and to optimise reproducibility of the experimental protocols.

We will also use our annual return data to estimate the number of animals that we will need to use in the future.

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

In order to optimize our experiments, we have used previous publications and pilot experiments already performed. We have optimized the techniques in collaboration with worldwide experts.

We have also consulted the Planning Research and Experimental Procedures on Animals:

Recommendations for Excellence (PREPARE) guidelines to ensure quality and optimize reproducibility of the experimental protocols.

We are also fully aware of the ARRIVE guidelines and the principles guiding the Replacement, Refinement and Reduction of animals in research (www.nc3rs.org.uk/ARRIVE/) and all experiments will be executed adhering to these principles.

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 maximize the number of readouts assessed per animal, thus reducing animal numbers required. Tissues that are not immediately analyzed will be stored for future analysis and may be made available to other investigators upon request.

All of the experimental protocols in the program have well-validated with robust end-points, which means that the expected spread of results for key end-points are already established.

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 Models: we will use mouse (*mus musculus),* spiny mouse (*acomys cahirinus*) and immunocompromised mice, at different ages. We have chosen well described models of regeneration and selected the best options for our experiments based on previous experience. For all the models, we have refined the endpoints to minimize the suffering (e.g., frequent monitoring via clinical score sheets) to ensure no animal suffers unduly.

Methods:

Skin transplantation: we use skin transplantation to understand the contribution of different skin cells to regeneration. We use microsurgical techniques that have been extensively refined to make them less invasive. We consulted with world-leading veterinary surgeons to improve suturing in order to make the recovery of the animal more comfortable. We are also in contact with plastic surgeons and dermatologists that have helped us to refine our methods based on their experience treating human skin.

Wound healing: we perform small superficial cuts in the skin to understand how healing occurs. This is a well-established model with a consistent analgesic regime. Cuts are superficial to preserve the vasculature, as this improves healing and minimizes pain for the animal. We also use dressing films to improve animal post-surgical care.

Paired-blood exchange: we are also interested in the contribution of circulatory factors to regeneration, so we extensively analyze mouse blood. We have also refined our blood extraction procedures (by placing vascular buttons that are connected to arteries and veins using minimallyinvasive surgery), allowing us to extract very small amounts of blood without piercing the mouse tail repeatedly.

Behavioral evaluation: ageing coincides with gradual and progressive changes in brain function and behavior. To address if our experiments ‘rejuvenate’ cognitive function, we will use non-invasive

behavioral tests, such as balance, event arenas and recognition memory paradigms. These are wellestablished tests that minimize animal suffering.

Ageing: animals may be held until 2 years of age to study ageing. Aged mice may display signs of ageing (e.g., cataracts) and may develop natural occurring diseases such as cancer. However, these are distinctly different from signs of ill-health. Should the mice present any signs of ill-health (pain, reduced social activity) they will be promptly humanely culled.

Sub-lethal irradiation: will be used to induce artificial ageing. This will allow us to explore the aged phenotype without maintaining the mice for two years, thus minimizing age-related conditions.

Administration of substances of interest (e.g., cells or drugs) may be administered to the animals to understand the biology of ageing. We aim to identify specific elements that can be applied to humans, to improve age-related conditions. We will use an appropriate route for administration and pilot studies to minimize risks.

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

Non-mammalian animals are limited in their use for this particular project as they do not present the same ageing response as human do. Skin composition (layers, cells, etc) is similar between rodent and human (with the difference of hair density and thickness), but completely different from less sentient species, which renders the later ineffective for this project.

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

For all protocols in this proposal, monitorization by ourselves and experienced staff in the animal facilities will be key to ensure any unexpected suffering is rapidly assessed and alleviated using analgesia, anesthetic or humane killing as appropriate.

We will use validated humane endpoints which trigger termination of a protocol (e.g., uncontrollable pain 48 hours after surgery).

Pilot studies will be used to substantially reduce the risks of any new experiment.

Skin transplantation and skin wound models: has been developed in collaboration with worldleading centers, optimizing the model for best results and animal care. Animals will receive analgesia to minimize pain during recovery. We will control all possible parameters during the surgery and recovery, improving aseptic technique through the use of specific sutures and dressings.

Blood-paired exchange: we have developed a highly refined microsurgical system to extract small quantities of blood without constantly pricking the mice. This allows us to study ageing circulatory factors.

Behavior evaluation: our refinements include intra and inter laboratory replicability tests, to avoid repeating the experiments unnecessarily and (when appropriate) use of video tracking-based automated quantifications to not disturb the mice.

Ageing: all animals > 1 year will be routinely subject to increased monitoring with regular weights recorded every two weeks along with full assessment of physical condition. We have extensive experience in monitoring old mice (unit staff and researchers).

Sub-lethal irradiation: animals will be closely monitored for weight loss and signs of post-irradiation immunosuppression. Monitorization will include 2 daily checks for the first 48

hours and will be subject to increased monitoring and regular weight checks weekly in the aftermath of irradiation. Animals could be treated with routine prophylactic antibiotics and analgesia as required.

7. Substances/Cell administration: appropriate route for administration will be determined based on literature and/or collaborations with other researchers. Pilot studies to determine minimum/maximum dose to cause the required effect will be used. Specific refinements will be included depending on route of administration, for example: topical administration will be used in combination with appropriate vehicles (e.g., petrolatum jelly) to avoid local inflammation. Administration of substances in the diet could be performed by mashing the diet and allowing easy access to hydration for the rodents.

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

We have consulted the Planning Research and Experimental Procedures on Animals:

Recommendations for Excellence (PREPARE) guidelines to improve the quality reproducibility of our studies. Protocols have been established in collaboration with centers of excellence to ensure optimization of the techniques and optimal care for the 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 and attend Regional 3Rs symposia.

We are also fully aware of the current 3Rs considerations when using ageing animals in science (which are summarized here: https://onlinelibrary.wiley.com/doi/epdf/10.1002/9781119555278.ch16). We have also set up regular alerts for any data release from the mouse ageing cluster in UK, to gather information about mouse ageing and any opportunities to apply the 3Rs.

26. Models of haematopoiesis, therapy, 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
- 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

Haematopoiesis & Haematopoietic stem cells, Gene therapy, Metabolic Disorders, Primary immunodeficiencies, Tumour therapy

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 project aims firstly to better understand how specific molecular problems lead to Primary immunodeficiency diseases (PIDs) and Inherited Metabolic Disorders (IMDs). By understanding these root causes better, we hope to improve the treatments that are already available and develop new therapies for these conditions.

Secondly, we aim to demonstrate pre-clinical efficacy and safety of cellular and gene therapy protocols and conditioning regimens developed in the laboratory before they are used in clinical trials. These methods include both cellular therapies (using cells from the body) and gene therapies (using genes to treat diseases) or a combination of both (using genetically corrected patients' cells). We want to make sure that these treatments are effective and safe before they are given to patients. Additionally, we aim to improve upon

the current methods used in clinical trials by developing better ways to deliver these therapies into the body and to specific tissues/organs.

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?

Primary immunodeficiency diseases (PIDs) are genetic conditions that weaken the body's ability to defend against infections. These disorders stem from flaws in the production of immune cells, which is a part of a complex process called haematopoiesis, and the way they function. Thanks to advances in medical technology, over 350 different types of PIDs have been identified, with new ones discovered each year. While primary immunodeficiencies (PIDs) can affect all ages, they are more commonly diagnosed in children. Most PIDs are rare, with an overall prevalence of approximately 1 in 10,000 live births. However, prevalence is significantly higher in populations with high rates of consanguinity or in genetically isolated groups and can vary across populations. People with PIDs often suffer from frequent infections, leading to a lower quality of life and, if left untreated, can be life-threatening. Similarly Inherited metabolic disorders (IMD) are fatal genetic disorders caused by mistakes in genes producing enzymes, which are needed for breaking down chemicals produced in the body. If enzymes do not work or are missing, toxic substances build-up in different body parts including the brain, where they cause severe problems with the way children think and develop. As for PIDs, Inherited Metabolic disorders (IMDs) are rare disorders more commonly diagnosed in children, however when considered all together, they may affect about 1 in 1,000 to 2,500 newborns.

Current treatments for PIDs and IMD can be costly and may have long-term side effects. For example, PEG-ADA (a current enzyme-based therapy for a PID) costs between £200,000 and £400,000 per year, while gene therapy offers a potential solution by providing a one-time treatment. This project aims also to enhance our understanding of PIDs and IMDs. Human samples are scarce, especially from affected children, making animal models essential for research. We also strive to improve treatments through advancements in cellular and gene therapies. Previous work has successfully transitioned gene therapy from experimental stages to clinical use, with mice models playing a crucial role.

Our research focuses on understanding and manipulating stem cells, and in particular haematopoietic stem cells, which are vital to produce immune and other cell types in PIDs patients and for the delivery of missing or malfunctioning proteins for IMDs and other rare diseases. We continuously develop methods to enhance new therapies (e.g. gene and cell therapy), aiming to improve their effectiveness and safety (e.g. with novel vector elements increasing expression levels and location). We expect further clinical trials to be started within the lifetime of this licence, with improved vector/gene editing strategy development showing promise for better treatment outcomes without severe side effects.

In the next 5-10 years, we anticipate further progress in treating PIDs and IMDs, and other rare diseases, using novel gene editing techniques/tools and refining vector technology based on a deeper understanding of the diseases at a molecular level.

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

The results of this research are pre-clinical data that will be used to translate novel cell and gene therapy protocols into the clinic. Additionally, we plan to share our findings through publications in scientific journals, which will benefit other researchers and the scientific community as a whole.

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

The main beneficiaries of this research are paediatric patients affected by monogenic disorders or cancer and their families. The knowledge accumulated with these studies will also shed light on the properties of different subpopulations of cells involved in haematopoietic system and immune system, such as haematopoietic stem cells or T cells which will be beneficial to the entire scientific community.

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

The outputs of this work will be used to secure further funding needed for translation of the pre-clinical data into the clinic. The data generated will be shared with the scientific community through peer reviewed publications and presentations at relevant scientific meetings, including unsuccessful approaches, in line with the principles of 3Rs.

Species and numbers of animals expected to be used

- Mice: 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.

We are interested in studying the disease mechanisms, and developing novel therapies for primary immunodeficiencies, inherited metabolic diseases and malignancies in a paediatric setting. Therefore, we have chosen to use immunodeficient models which are highly efficient in engrafting human haematopoietic and immune cells. Moreover, the mouse models we use faithfully reproduce most of the clinical signs and phenotypes observed in humans. In general, we will use adult mice for most of the experiments. However, there may be a few instances where neonates and embryos may be studied, to specifically understand the pathogenesis around the onset of disease as seen in human neonates and the influence of a specific mutation in the perturbation of normal development of the haematopoietic and immune system.

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

Animals will be bred, tagged, marked, and genotyped as appropriate. They may be used only for harvest of organs to study a particular disease phenotype at a particular stage of their life or may be used in other experiments. Animals may be used in transplantation studies after conditioning by Chemotherapy, irradiation or antibody-based reagents. Transplantation may be carried out by intravenous, subcutaneous, intraperitoneal, intrafemoral routes in adults, or intrahepatic or supra-facial veins in neonates, or in utero laparotomy for transplantation into embryos. The engraftment of the transplanted donor

cells will be tested by peripheral blood sampling or bone marrow biopsies of the recipient mice. The effectiveness of the immune reconstitution may be tested by challenging with immunomodulators. Animals may be then sacrificed by a schedule 1 method or by terminal anaesthesia for organ harvest. Animals transplanted with cell and gene therapy products may be functionally assessed using motor function tests, cognitive/behavioural tests, and scans to confirm the restoration of their normal function. These tests are especially relevant to metabolic disorders, which are complex multisystem disorders that may exhibit musculo-skeletal, brain, cardiac and respiratory abnormalities. Induced pluripotent cells, and their derivatives, may be assessed for their safety and efficacy by transplanting them into immunodeficient mice. Occasionally certain induced pluripotent cells may induce tumours, and those animals will be sacrificed humanely before it exceeds the protocol severity. Gene and cell therapy products which are specifically developed for treating tumours will be tested for their anti-tumour efficacy, after transplantation of the minimum number of tumour cells required to form a tumour. The time taken for the development of tumour may range between couple of weeks to a few months depending on the type of tumour studied.

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

Immunodeficient mice may develop pathogenic infections. Animals are therefore maintained in individually ventilated cages with sterilised bedding, water, and food. Inherited metabolic disorder mouse models may occasionally develop signs of joint/muscular disease, respiratory, central nervous system, or liver disease. Where possible mice will be treated before the onset of disease rather than once disease has been established to minimise suffering. Animals that are conditioned using total body irradiation are expected to experience some weight loss, loss of condition during the first few days after irradiation and, in some instances, mice might develop teeth issues around 5 to 8 weeks postirradiation. To limit these, damp diet or food supplement will be given after conditioning, while weight will be carefully monitored. Pregnant dams that have had laparotomy for in utero transplantation of embryos are expected to suffer moderate post-operative pain for a few days. Transplanted mice that undergo bone marrow biopsy are expected to experience moderate pain and transient difficulty in ambulation for a few hours. Superficial blood vessel sampling can (very rarely) cause profuse bleeding. Induced pluripotent stem cells, upon transplantation can induce malignant transformation, and therefore some animals may form tumours, which can cause moderate pain. These tumours develop at the site of sub-cutaneous injection in flank region and is not expected to cause significant disruption to their normal activity. Animals that develop tumours are usually culled within a few days from the initial diagnosis. Animals that are subjected to motor and behavioural functional tests may experience some mild discomfort for a few minutes during each time point of testing. Animals that are transplanted with tumour cells are expected to show weight loss, loss of appetite and general wellbeing for a few days, which may cause moderate pain. These symptoms usually relate to the tumour volume, site of tumour formation, degree of metastasis etc. On very rare occasions, superficial tumour may bleed or ulcerate and can cause secondary infections.

Mice undergoing any procedure will be closely monitored and given analgesics and appropriate treatments to relieve pain and mitigate any anticipated 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)?

Most of the animals do not suffer from significant adverse effects from the procedures that are undertaken in this license. However around 10-20% of the animals may suffer some from some clinical signs for a short period of time, as a result of the underlying disease.

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?

Primary immunodeficiency Diseases (PIDs) and Inherited Metabolic Disorders (IMDs) are very rare diseases, and patients are few (even at large centres like Children's Hospital). Affected individuals are often very young children from whom only small blood samples can be obtained. The use of animal models of these diseases also allows us to study the immune system in vivo which is often not ethical or practical in humans. No other non-animal models exist that mimic human immunodeficiency and metabolic disorders and allow xenotransplantation as well as the PID/IMD mouse models we want to use. The complex organisation of the haematopoietic system makes in vitro culture unsatisfactory. In vivo experiments are required to measure engraftment and longevity of haematopoietic stem cells and development of functional immunity because the complex multifactorial interactions cannot be assessed in vitro. The safety of vectors over long periods of time and the potential for insertional mutagenesis can only be adequately assessed in vivo. In fact, the current guidelines state in vivo testing is mandatory for testing efficacy of treatment in disease models, and assessing safety for the whole organism. It is not ethically possible to study PIDs, IMDs, and more generally haematopoiesis or conduct cellular and gene therapy protocols directly in humans and current in vitro techniques are not sufficient for research or clinical regulatory bodies. In vivo experiments are required to measure engraftment and longevity of haematopoietic stem cells and development of functional immunity because the complex multifactorial interactions cannot be assessed in vitro. In addition, we want to assess the impact on health and behaviour. The safety of vectors over long periods of time and the potential for malignancy can only be adequately assessed in vivo.

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

In vitro cultures

Ex vivo 3D models

Mathematical modelling

Why were they not suitable?

In vivo experiments are required to measure engraftment and longevity of haematopoietic stem cells and development of functional immunity because the complex multifactorial interactions cannot be assessed in vitro. In addition, we want to assess the impact on health and behaviour. The safety of vectors over long periods of time and the potential for malignancy can only be adequately assessed in vivo. We are currently employing advanced in vitro and ex vivo experimental approaches, such as organoids and microfluidics, to closely mimic the in vivo environment. These methods provide crucial insights and help us identify potential targets to further investigate in subsequent in vivo transplantation experiments. However, definitive evidence of hematopoietic stem cell transplantation/hematopoietic stem cell gene therapy efficacy can only be achieved through in vivo transplantation studies. This is primarily due to the complexity of the hematopoietic system and the signalling interactions from the hematopoietic stem cell niche, haematological tumours, and, in cases of neurological metabolic disorders, the central nervous system. Even the most sophisticated in vitro systems cannot fully replicate the interactions between hematopoietic stem cells, their niche, and other relevant cell types within the neurovascular unit and brain, along with the complex signalling unique to the in vivo environment. In fact, the current guidelines state in vivo testing is mandatory for testing efficacy of treatment in disease models, and assessing safety for the whole organism.

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 each experiment involving mice we will use statistical software packages and power analysis in order to define the minimum number of mice to be used as well as using our knowledge from previous experimental group numbers. Number of animals in each group will be adjusted depending on the effect size expected. Typically, we expect experimental groups to consist of six to twelve mice. Sharing control groups for comparable treatments groups will also reduce the number of mice to be used.

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

Careful considerations were given to the Arrive guidelines and information available through the NC3Rs website using features such as Experimental Design Assistant to ensure that the minimum number of animals are used in this project keeping in mind the experiments are robust and reproducible as possible and providing the best welfare possible for the animals.

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

Where possible, each animal will be used for multiple analyses post-mortem (for example histological and immunological). Where animals must be genotyped, this will be done as early as possible and wild-type littermates will be used as controls thus minimising the

need to purchase control animals. For longitudinal studies such as behavioural studies, we will maximise the observation points during the period when clinical signs are expected to reduce the overall number of animals 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.

We have chosen mice as we are studying models of inherited disease and mice are the most appropriate and best characterised lowest vertebrate group animal model. Murine models are regarded as the most appropriate judged by peer reviewed publications. Immunodeficient models are the most appropriate models which will allow the engraftment of human cells that are subjected to gene therapy and gene editing protocols.

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

Where appropriate, we use neonatal mice for studying haematopoiesis, by engrafting gene therapy and gene edited cells via intra-hepatic or supra-facial route in neonates and by in utero approaches by directly transplanting into foetuses. However, some of the disease phenotypes are exhibited at certain developmental stages of the animal and often only in mature animals, requiring investigation at different time points.

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

Where possible we propose to treat before the onset of disease with in utero injections rather than treat mice once disease has been established to minimise suffering. We have experience with breeding immunodeficient and metabolic disorders animals, and with long established protocols that have well defined end points, so we can provide high level training and thus competence for experimental procedures. Where necessary we will seek out advice and expertise from other groups in the UK and abroad to minimise suffering.

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

The ARRIVE and PREPARE (http://journals.sagepub.com/doi/full/10.1177/0023677217724823) Guidelines

NC3Rs and Laboratory Animal Science Association resources

Workman et al, British Journal of Cancer 102, 1555–1577 (2010) guidelines for tumour studies

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 (https://www.nc3rs.org.uk/), attend regional 3Rs symposia and external conferences and seminars on animal welfare.

27. Zebrafish models of human congenital ocular malformations

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 zebrafish, ocular malformations, eye embryogenesis, CRISPR-Cas9 approaches

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?

Our primary objective is to advance our understanding of the mechanisms controlling eye formation, and to identify new genes important for eye formation and involved in eye malformations in 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?

Eye malformations are a main cause of childhood blindness and occur when genes controlling eye formation during pregnancy are defective. However, defective genes have only been identified in 30% of affected patients, meaning that in most cases, the genetic cause of the defects seen in these children are unknown. One tool to identify novel genes are animal models with defects in genes suspected to be important for eye formation. Our primary objective is to generate genetically altered zebrafish strains in candidate genes identified from our research, and study them to better understand the function of those genes and how might they lead to eye malformations when defective.

The data generated over the life of this project licence will lead to the identification of new genes involved in promoting human congenital eye malformations when disrupted. The inclusion of these new candidate genes in screening panels for ophthalmic conditions will expand the number of identified causes of congenital eye malformations, and improve our diagnosis approaches. Additionally, this programme of research will expand our knowledge of the genes involved in eye formation in normal conditions, an essential pre-requisite to understand the mechanisms involved in eye disease. In the long term, this research will therefore benefit society by improving diagnostic strategies and genetic counselling for the affected patients. Other researchers in our field will also benefit from our studies, which will build up our overall knowledge of eye formation.

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

Our research will identify new genes involved in promoting human congenital eye malformations when disrupted. Additionally, our research will expand our knowledge of the genes involved in eye formation in normal conditions, an essential prerequisite to understand the mechanisms involved in eye disease.

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

This project will identify new genes to be considered in the aetiology of human congenital eye malformations. Our studies generate new basic knowledge and have several main beneficiaries: other researchers in the field, affected patients with ophthalmic diseases, and the wider society.

In the short term, our studies will have an immediate impact on our overall knowledge of eye formation and mechanisms of embryonic development. The new genetically altered strains of zebrafish that we may generate will be made available to the wider research community, and will be useful tools not only for ourselves, but also for other researchers, to expand our understanding of the genes involved in eye formation.

Basic research findings may need between a few years and decades to reach clinical practice. The impact of our studies on better diagnosis may thus not be immediate. Despite this, our studies will be essential to inform clinical practice and we expect they will have a long term impact, beyond the life of the project. Genes identified by our studies will constitute new candidates to be included in screening panels for ophthalmic conditions. This will expand the number of identified causes of congenital eye malformations, improving our diagnosis approaches and genetic counselling for the affected patients.

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

We maintain long term collaborations with other researchers in the field. As part of these collaborations we have also links with clinicians in the ophthalmology field, ensuring a route of communication of our basic research findings to the clinical practice. In addition, we share expertise and resources with other groups at our institution, where we form part of a well established group of researchers focused on understanding the cell biology and the genetics underlying a variety of human congenital diseases.

An essential part of scientific research is the communication of our studies to colleagues in the field. We will maximise the dissemination of our results (both positive and negative) by participating in national and international conferences in our field of research, where we will present our studies prior to publication. The results from our work will be subsequently published in peer reviewed, open access scientific journals. Our institution has signed the San Francisco Declaration of Research Assessment (DORA) and maintains a public access repository in which all our publications will be stored and made available to the

wider research community. We actively participate in outreach events at our institution, and maintain an up to date institutional website in which the aims and results of our research are regularly updated.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 6050

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 make use of the zebrafish as a model system. Our choice of animal model allows us to keep the number of adult animals used to the minimum as compared to other vertebrates. Indeed, zebrafish embryos are fertilised externally and one pair of adult fish generates hundreds of embryos per week.

This allows us to perform extensive phenotypic analysis in large numbers of embryos, without the need to sacrifice the mother. We will use a variety of approaches to analyse the function of genes important for eye formation in our animals. Some of them will constitute regulated procedures, but we have refined our approaches so that as much of the analysis as possible will be done on embryos prior to 5 days post-fertilisation (dpf), and will thus constitute unregulated procedures. This will ensure that we use the minimum number of mature animals possible and perform the minimum number possible of regulated procedures.

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

We expect to generate several new genetically altered zebrafish strains over the life of this project. Some of these lines will carry mutations in the candidate genes selected over the course of our studies, and others may carry an exogenous transgene. We expect that the genes we are analysing will be involved in the formation and maturation of the visual system. Thus, mutations in those genes in homozygosis will be expected to lead to eye defects or visual deficiencies and blindness. Adult animals carrying these mutations in heterozygosis will be bred to maintain the colonies of genetically altered animals necessary for the progression of the project, and to obtain embryos for analysis.

We have refined our experimental approaches so that most of the procedures to be performed in the course of the project will require phenotypic analysis prior to 5 dpf and will thus not constitute regulated procedures. These embryos will be euthanised and subsequently will be treated for tissue analysis. Occasionally, we may grow animals that do not show any observable phenotype during embryogenesis beyond 5 dpf, to determine whether eye phenotypes develop as the animals mature. These animals will be carefully monitored and phenotypic analysis will be done at the earliest timepoint possible to detect visual deficiencies and always before they reach the humane endpoint. We will keep these regulated procedures to the minimum and the animals will be euthanised upon completion of the test.

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

Regulated procedures in this project will involve generation of new genetically altered strains, genotyping of adult animals, or visual acuity tests to assess visual performance of larvae.

Genetically altered strains of fish produced under this project will carry mutations in the candidate genes selected over the course of our studies. We expect that the genes we are analysing will be involved in the formation and maturation of the visual system. Thus, mutations in those genes in homozygosis will be expected to lead to eye defects or visual deficiencies and blindness. In some cases, ocular defects will be detected in embryos under 5 dpf, and functional analysis of those strains will be done at those stages and will thus constitute unregulated procedures. In other cases, genetically altered animals may not show any observable phenotype prior to 5 dpf. In those cases, we will grow the animlas beyond 5 dpf, and closely monitor them to determine the earliest timepoint at which eye phenotypes can be identified. Visual performance tests will be done on animals at 7-8 dpf, allowing us to identify visual deficiencies in those animals that do not show observable morphological eye defects. A subset of the animals may be grown to adulthood to examine eye tissue integrity and progression of the phenotype as the animals age.

Some fish may have the potential to develop an unexpected harmful phenotype after a certain age.

Fish exhibiting any unexpected harmful phenotypes will be euthanised, or in the case of individual fish of particular scientific interest, advice will be sought promptly from a Home Office Inspector and the Named Veterinary Surgeon. The genotyping procedures and visual protocols in place are well established and extensively refined, and are not expected to have a significant negative impact on the wellbeing of the fish subjected to it. Measures have been put in place to monitor and assess any unexpected adverse effects, and animals will be euthanised in those cases where they are detected.

In all cases, the health and wellbeing of all fish over 5 dpf will be regularly monitored and they will be euthanised at the first sign of 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)?

Most of the animals will experience a subthreshold or mild severity. A small proportion of animals (up to 5%) may experience a moderate severity, if they develop unexpected harmful phenotypes after a certain age. Some of these animals will have been exposed to small molecules prior to visual tests that may temporarily cause a moderate effect. To avoid any suffering fish will be closely monitored for signs of unexpected harmful phenotypes and immediately euthanised if any is detected.

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 project outlined in this proposal aims at understanding how the eyes form during embryogenesis, and to search for genes that when affected lead to eye disease. The nature of this work requires the use of whole animals. Indeed, visualisation of the processes leading to eye formation, which involve complex interactions between cells with different embryonic origins and results in an organ with a complex three-dimensional structure, can only be performed in an intact organism. In addition, the recapitulation of disease features in congenital eye malformations can only be accomplished in a whole animal.

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

In recent years the use of in vitro models of organogenesis called organoids has been greatly improved, and it is now possible to develop rudimentary optic cups that reproduce some of the main features of an embryonic eye. These in vitro models, however, do not include all the cell types of a normal eye, and some of the tissue specialisations affected in the diseases we study (such as the high acuity area in the retina or the choroid fissure) are not accurately reproduced in them. Thus, these models cannot replace the use of animals for the studies we perform.

To optimise our approaches and ensure that we minimise the number of animals we use, we perform a detailed literature review at the start of each of our studies to inform our project and ensure that we do not duplicate previous work in the selection of candidate genes. This allows us to keep to the minimum the number of animals we need to use for our research.

Why were they not suitable?

The generation of a fully functional eye requires the complex interaction of multiple tissue types, and thus is currently not possible to use organoids as a substitute to animal work in our studies. Instead, the right choice of animal model and the careful review of previous research allows us to keep to the minimum the number of animals we 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?

In the course of the project we will make use of several strains of fish with different modifications in their genome. The use of these organisms is essential for the progress of this project. Adult fish will be maintained for the purpose of obtaining the embryos required for the research, and occasionally to determine if any visual deficiencies arise as the animals age in some of our conditions. Animals enough to maintain one generation of breeding adults per strain at any time will be kept. From previous experience, we estimate this number to be around 15 adult breeding animals per generation. New generations will be grown from heterozygous carriers when breeding adults reach 12-18 months of age and generations will be replaced every 18 months. Since we need to genotype the newly bred animals to determine their genetic status when they reach maturity, and not all of them will carry the mutations, we need to raise an excess of animals until genotyping can

be performed. Thus, at any given time we expect to have between 60 and 80 fish (between juveniles and adult breeding fish) per strain.

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

We have refined our approaches so that most of the procedures will generate embryos for analysis prior to 5 dpf. A pair of healthy breeding zebrafish adults produces over a hundred embryos per mating, providing us with substantial amount of material to perform embryonic manipulations. When derived from a strain bearing a mutation, pools of embryos comprising normal and affected embryos will be treated and analysed all together, ensuring a reduced bias and uniformity in the experimental procedures.

For those procedures to be performed in protected animals, we will determine the number of animals to be analysed with the help of the NC3R's Experimental Design Assistant and consideration to the estimated proportion of animals in a mating that will carry the relevant genotype.

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

We do detailed literature reviews and analysis of previous research to inform our project, and to ensure that our selection of candidate genes does not duplicate previous research. We will only generate new genetically altered strains when these are not available in the research community. We will cryopreserve those strains that are not actively used anymore, ensuring we maintain the minimum number of live animals at all times.

We are always striving to identify new approaches to refine our experimental design and reduce the number of animals used. As part of this continued experimental development, we are piloting the use of the Zebrafish Embryo Genotyper, and the genotyping from embryonic fin biopsies. If optimised, these genotyping approaches will allow us to grow to adulthood only the animals carrying the relevant genetic modifications and will greatly reduce the number of animals used under this 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.

Most of the experiments described in this project licence will be performed on zebrafish embryos not older than 5 dpf. Adult fish will mostly be generated for the purpose of obtaining the embryos required for the research. Our choice of animal model allows us to keep the number of adult animals used to a minimum as compared to other vertebrates, since zebrafish embryos are fertilised externally and one pair of adult fish generates hundreds of embryos per week.

Mild procedures may be performed on the fish if genotyping of the adults is required. In these cases, animals subjected to a procedure will be provided with adequate analgesia

pre- and post-procedure and closely monitored for recovery. Once the animals are not needed anymore, they will be euthanised using an established procedure.

The visual acuity tests that will be performed on larvae over 5 dpf are not expected to cause any harm to the animals. In some cases, animals will be exposed to small molecules and a proportion of these may temporarily cause a moderate effect. Animals will be carefully monitored during the procedure and euthanised after the procedure.

We expect to generate several new zebrafish strains with genetic modifications over the life of this project. These strains will be maintained in heterozygosis, and we expect no adverse effects to show in them. Occasionally, homozygote embryos not displaying any observable phenotype during embryogenesis may be grown to adulthood to determine whether eye defects arise as the animal matures. In these cases, animals will be closely monitored for signs of unexpected harmful phenotypes. Fish exhibiting any unexpected harmful phenotypes will be euthanised, or in the case of individual fish of particular scientific interest, advice will be sought promptly from a Home Office Inspector.

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

We have selected the zebrafish as the least sentient animal we can use for our studies, which require modelling human ocular malformations to understand their mechanisms and aetiology. In addition, we have refined our approaches so that most of the procedures will generate embryos for analysis prior to 5 dpf therefore avoiding the use of large numbers of protected animals.

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

Adverse effects in our animals will be scored by looking at growth rates and swimming patterns. Any juveniles that show a significant difference in size to their siblings, or aberrant swimming patterns, will be closely monitored and euthanised if sign of suffering is detected. The health and wellbeing of adult fish will be regularly monitored and they will be euthanised at the first sign of suffering.

Any animal subjected to mild or moderate procedures will be provided with adequate analgesia.

Staff responsible for animal handling will be appropriately trained to ensure the best handling of the animals.

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

We regularly consult the NC3rs newsletters and make use of the NC3Rs library of resources to design our experiments. In addition, we will keep ourselves updated on the recommendations presented by FELASA for zebrafish research.

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

We will maintain continued communication with the staff in our animal facility, and follow their advice and suggestions regarding replacement, reduction and refinement of our experimental approaches. We will keep ourselves informed about any new advances by regularly consulting the NC3Rs website, and the recommendations presented by the Federation of European Laboratory Animal Science Associations (FELASA) for zebrafish

research. Our facility has appointed a 3R's Champion who reports to every AWERB meeting and new advances are regularly communicated to users. As part of our dissemination strategy we participate in conferences and meetings in our field of research. Conferences such as the European Zebrafish Meeting, the European Principal Investigators Meeting and the International Zebrafish Conference allow us to keep up to date with the latest advances in zebrafish husbandry, welfare and research. In addition, we are active members of our local community of zebrafish researchers in the London area, and regularly participate in meetings where best practice is shared amongst research groups.

28. Unravelling disease mechanisms in motor neuron disease and 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

Motor Neuron Disease, Dementia, Cell function

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 project aims to improve our understanding of how 'good proteins go bad' in motor neuron diseases and dementia, and to try to learn what processes in the cell go wrong and identify if fixing any of these processes may help treat 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?

Motor neuron diseases and dementia are devasting disorders with no current cure, or truly effective treatments. As diseases of aging, their incidence is growing, placing a significant socioeconomic burden on society. Despite several decades of research, the quest for effective treatments has remained elusive. One key reason for this is the relative complexity of the disease process, with many different factors that appear to contribute to disease onset and progression. Developing an improved understanding of how our proteins work and interact in healthy cells, and how this differs in disease, is crucial in

understanding what are the core drivers of disease. This in turn will allow us to identify and evaluate potential new targets for drug development to treat these disorders.

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

Outputs will include new information and research publications detailing the role of key proteins in the disease process, and unpicking what part of some of these proteins may be most important in disease. In addition, this project may provide preclinical validation for potential novel therapeutic targets or compounds, paving the way for therapeutic development or a transition into clinical trials.

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

In the short term, other researchers in the field of neurodegenerative diseases will benefit from these outputs, as well as researchers across the broader spectrum of neuroscience research. In the longer term, the successful identification of new therapeutic targets for drug development, or the preclinical validation of prospective compounds for progression to the clinic has the potential to benefit patients and their families, as well as doctors, social care and other workers and care networks who support these patients.

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

This project will be run in collaboration with several colleagues to ensure engagement and sharing of all findings at the earliest possible times, and hence prevent unnecessary duplication of research and ensure all work carried out under this licence has robust support from in vitro studies. In addition, work will be disseminated in scientific publications, and shared at national and international conferences, and across relevant research networks, to ensure that relevant findings reach the broader scientific community.

Work will also be shared on websites such as Biorxvii, to allow relevant unpublished data to also be shared with the research community, and successful and unsuccessful research approaches and 3Rs improvements will be shared within our local in vivo research network, and disseminated to the wider UK animal facilities and researchers via dedicated 3Rs engagement sessions, which are currently planned annually at our institute.

Species and numbers of animals expected to be used

- Mice: 7600

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 is aimed at understanding more about what causes the age-related disorders of motor neuron disease and dementia, specifically how different protein and cell functions are altered in disease, and whether repairing these defective pathways can be beneficial. One brain region that is more heavily affected by these diseases than others, is called the cortex, which is only found in mammals, hence we need to use a mammalian model to understand what happens specifically in the cortex in order to understand the disease processes.

In addition, the impact of motor neuron disease and dementia is seen across complex networks of different types of brain cells, and occurs with aging. Both these factors we cannot currently model effectively in vitro.

Finally, key read outs of these diseases are the learning, memory and movement impairments suffered by patients. To find out what aspects of the disease biology are involved in these processes, and explore whether modifying specific proteins or drug targets may provide symptom relief, or slow or prevent disease progression, we need to be able to measure these in our studies.

Taking these factors together, the use of adult and aged mammalian models of disease is the optimal way at present for us to address the complex biological and therapeutic challenge presented by these devastating disorders.

The mouse has been selected as the best model for a number of reasons, most importantly because there are a wide array of pre-existing mouse models of motor neuron disease and dementia, many with well established disease characteristics, and clear time frames for disease development. In addition, the behaviour of healthy mice in a spectrum of relevant behavioural tasks is well established, as are their welfare requirements. The use of adult and aged mouse models therefore allows us to carefully plan and design our studies to minimise adverse impacts to the animals, and keep numbers as low as possible.

In rare cases, we may allow mice to age up to 30 months. In humans, many of these diseases do not occur until old age (60-80 years old). While we expect disease processes to be accelerated in our mouse models, in some cases things still progress slowly with symptoms being mild or non detectable even out to two years old, hence occasionally we may want to investigate changes in very old animals. Using these very old animals should allow us to identify clearer signs of the disease to relate changes in the model back to the human disease. This will enable us to be more confident of which earlier cell changes we see are important in the disease process, to give us clearer readouts in younger animals for subsequent studies.

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

In most cases (~70-80%), animals with genes that give them aspects of a neurodegenerative disease as well as healthy controls will be allowed to get old (typically no more than 24 months, although in rare cases (<5%) to a maximum of 30 months) and develop disease, and their brains, spinal cords and in some cases muscles will be collected. Some of these animals (up to 50%) may also be assessed for movement or memory changes as they age using well-established tests such as the rotarod test for movement, and the Morris water maze for memory. Some of these animals (<10%) may be exposed to a single or repeated (max 5 times across their lifetime) 90-120 min heat exposure (being placed in a chamber with air temperature of around 40^{o}C) designed to temporarily raise core body temperature and mimic a fever at selected time points throughout the aging process.

Finally, the remaining 20-30% mice will be used for potential therapy assessment. These animals will be both healthy controls and those with genes that give them aspects of a neurodegenerative disease. These mice will be treated with the treatment or a control either via a single or repeated injection/oral administration (typically no more than once a day for 12 months) or via a direct surgical injection into the brain/spinal cord (once only). These animals will be allowed to get old (no more than 24 months) and develop disease, and their brains, spinal cords and in some cases muscle will be collected. Most of these

animals will also be assessed for movement or memory changes across the course of their lifetime.

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

For most animals on this project, the expected impacts will be movement or memory problems that develop as they age, and are associated with the development of the disease we are modelling. For animals with memory problems, they may display reduced grooming behaviour, but no other specific adverse impacts are expected. For animals with movement problems, these are not associated with pain, but are expected to worsen over the life time of the animal, In most cases this movement impariment will not significantly impact their ability to move about their home cage. A small number of animals may have difficulties in moving their hind limbs, and show some evidence of intermittently dragging these limbs. This is only expected to occur in the very late stage of the disease, lasting no more than 1-2 weeks, and if it progresses into sustained dragging the animals will be culled. Coupled with mild movement difficulties, animals may show evidence of weight gain, especially as they get older (18+ months of age). If the movement impairment is more severe, animals may show evidence of weight loss as the disease progresses. Any animals expected to develop more severe disease will be provided with moistened food at floor level, as well as longer water bottle spouts to help them access food and water. Once weight loss is evident, it is expected to progress and any animal showing evidence of general ill health or 15% weight loss will be immediately culled. This is anticipated to occur within 1 week of weight loss being observed.

Aging per se can result in adverse impacts in mice. A small number of old animals may develop tumours, or other signs of generalised ill health unrelated to the specific disease being investigated. If these effects are having an adverse impact on the animal, it will be immediately culled. Aging and long term maintenance of animals can also result in excessive grooming behaviour and/or scratching (leading to fur loss and occasionally injury) or fighting (typically in males). If impacts result in discomfort or noticeable stress to the mice, steps will be taken to treat them (eg treating wounds, separating fighters, trimming nails), and animals are expected to be recovered within a maximum of 1-2 weeks. Any animals with more severe injuries will be culled.

Animals undergoing heat stress could experience acute discomfort as a result of the heat. This is not expected to last more than 30min after removal from the heat.

Animals undergoing brain injections under anaesthesia could experience acute discomfort as a result of the surgery. This is not expected to last more than 24h.

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: 60% mild, 40% 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 project aims to learn more about the complex processes that lead to neurodegenerative diseases such as motor neuron disease, frontotemporal dementia and Alzheimer's disease. These diseases affect several different types of cells within the brain and spinal cord, with lots of different processes in these cells going wrong. Importantly, some cells in the brain appear more resistant to disease than others, and understanding why this is, and which aspects of cell dysfunction actually contribute to the disease process will provide valuable information that may lead to new types of treatment for these devastating diseases. The complex nature of these diseases, and the different cell types involved, means that it is difficult to create accurate models of the cell networks that are affected in disease. It is very likely that the environment the cells live in is just as important to their health as their own behaviour, and at present, the only effective way we have to model this environment is in live animal models. In addition, these diseases occur as we age. Brain aging is complex and poorly understood, and in vitro models are currently poorly equipped to model this process.

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

We have used (and will continue to use) various cell models (grown in a dish). This includes stem cells, and also developing more complex models that incorporate more than one cell type.

We also use brain slice cultures, which are thin slices of brain (and spinal cord), that we can grow in a dish.

Why were they not suitable?

There are several reasons that these models cannot fully replace live animal models at present:

They are not yet able to accurately replicate the complex and diverse cell make up of the brain, and its billions of connections. This is likely very important in what makes some cells more susceptible to disease than others.

They lack many of the environmental factors that cells in our brain are exposed to, both locally, from nearby cells and connections, and also more globally, as a response to environmental changes, such as hormones. This may be very important in understanding why some people are more likely to get disease than others, especially when we are trying to understand how our environment might influence our disease risk.

They lack the capacity to age in the same way we observe in the brain. Aging is a core risk factor for neurodegenerative disease, so accurately reflecting how cells change with age is crucial for understanding what might go wrong in disease.

Clinically relevant changes, such as alterations in movement or memory, are key features of neurodegenerative diseases, and these can only be measured in living, behaving 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?

Numbers for use in this prject have been estimated as follows:

To assess how aging and protein/protein pathway function may interact and play a role in disease, numbers have been selected based on our experience with pre-existing mouse lines that are expected to behave similarly, along with an estimation of the number of new genes/proteins/variants we are likely to want to investigate over the course of the project, and how many different questions we want to address. We have taken into account any likely ability to use samples from one mouse to address more than one question.

For therapy assessment, numbers have been estimated based on total numbers required for the validation of a single therapy/target, together with an estimation of the likely total number of different targets and disease models we are hoping to test over the course of the project.

For the heat stress experiments, numbers have been estimated based on our preliminary work in trying to establish this model, together with findings from us and others using cell and slice culture models, to gauge what our optimal experimental approach is likely to be, Then for the follow up longitudinal study, we have based numbers on both this information, and our experience with the disease model lines we plan to use for this study.

In addition to this, we have accounted for the number of animals required to maintain our different lines of genetically modified mice thoughout the course of 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, appropriate searches were done to identify which mouse models might be available for us to use, and how well suited they would be to the specific questions being asked. We also ensured we had access to all available information on relevant models to ensure we could design our read out time points to maximise the information we would get while aiming to keep animal numbers low.

Where relevant, we have integrated additional cell and slice culture studies into our experimental design prior to moving into live animal studies, to allow us to reduce the total numbers of animals needed, and only progress the most informative studies forward into animals. This includes work involving specially designed mutant models to address specific questions; all initial studies in this area are being carried out in cells. This will allow us to address only the most relevant questions in live animals, and minimise animal use.

Consideration has also been given to the amount of variation we see between control animals for each factor to be measured. This is based on our own experience in various models, as well as reports by others in the literature. Extra consideration was made for the variables that we can control, including sex, age and background strain. The statistical approaches to be used at the end of the study formed a major part of the experimental

design process, ensuring that all research approaches are robust and as unbiased as possible, while minimising excess animal use.

Experimental design was also discussed with other researchers familiar with these kinds of studies, to further validate the design and ensure we had included appopriate and adequate control groups for each study, such that all data obtained will be valid.

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

As far as possible, all experiments will be conducted longitudinally, to allow us to maximise the data obtained from each animal. For tissue harvest, all organs of interest will be harvested from each animal, and experiments will be designed to allow multiple follow ups in the tissue from each animal, thus minimising the total number of animals required.

Where relevant, and in most cases of therapeutic testing and initial heat stress induction experiments, 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, without any unexpected adverse effects, and also to allow subsequent experimental design to be optimised to use the minimal number of required animals. This will ensure that unnecessary large scale experiments do not take place.

As far as possible, breeding strategies will be designed so that all animals from a mating are used for an experiment, and for general maintenance of a line, animal breeding will be monitored and controlled to ensure that we obtain sufficient mice to maintain the line, while minimizing the birth of mice that are not 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.

All work planned in this project will use mice. This includes healthy control mice, and mice that contain genes that give them aspects of various neurodegenerative diseases including motor neuron disease, frontotemporal dementia and Alzheimer's disease. Since these are all diseases of aging, animals are healthy when they are young and develop memory or movement problems as they get older. None of these models result in any pain for the animals, but some animals will develop movement problems. These problems are progressive, so animals can be monitored for any decline, and additional care measures can be implemented to minimise distress if required. Experiments will be designed to ensure that these animals do not experience any significant long-term discomfort as a result of these movement problems, and in most cases, disease models that only develop mild mobility issues will be used. Aside from specific disease models, other mouse models will contain carefully designed alterations to proteins known to be important in disease, to allow us to understand the contribution of these proteins to disease. All these models are designed to have subtle impacts on protein function, and are not expected to have significant negative impacts beyond those seen in the disease models.

The motor and memory tests to be used in this project are all well established tests, with no anticipated adverse impacts on the mice, and animals will be monitored throughout the testing process, to ensure they do not experience any distress.

Surgical injection into the brain is a procedure that is required for some of our therapy treatment approaches, to ensure our treatments reach their target sites. This procedure is minimally invasive, with animals maximally receiving a very small hole (~1mm diameter) into the skull, and animals are usually completely recovered from this within a week.

Repeated injections into the periphery to administer treatments could cause some discomfort in mice if repeated for many days. As far as possible, we will minimise the length of time these treatments are required for, and aim to deliver longer term treatments in the food or drinking water if possible. We will also alternate injection site as much as is viable to reduce the risk of any long term tenderness building up. If we need to administer doses via oral gavage, we will use soft flexible tubes to minimise discomfort, and allow the mice several days to get used to the process prior to the start of the study.

To induce heat stress, some animals will be placed into an environment with a temperature higher than their body temperature. This should result in a rise in their core body temperature similar to that seen during a fever. This exposure will be acute (2h max), and is not expected to result in any long term problems for the animal. They will be monitored closely throughout the procedure, including regular measurement of their body temperature, to ensure that it never rises above safe levels.

A small number of healthy control mouse pups will be used to provide brains for cells and brain slice experiments in place of live animals wherever this is possible. The use of this model will ensure that animals do not experience any distress or suffering as a result of the experimental aims.

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

This project is designed to understand how different cell processes may be important in the onset and progression of neurodegenerative diseases such as motor neuron disease, frontotemporal demental and Alzheimer's disease. These diseases affect the brain, a highly complex organ made up of many different cells, and billions of connections. Specifically they affect a region of the brain called the cerebral cortex, which is only found in mammals. In addition, these disorders are progressive, and develop as we age, and are associated with changes in movement and memory. To be able to fully understand what goes wrong in disease, and try to design ways to prevent or stop it, we need to be able to see the disease progress, and examine changes at multiple stages. At present, mammalian models such as the mouse provide the optimal way to achieve this. Since aging is a key contributory factor to these diseases, we need to be able to study the adult and aging brain, hence we cannot use younger or neonatal brains for this work.

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

All animals undergoing brain injections will be given a long acting pain killer and local anaesthesia to minimise surgery associated pain. They will be monitored for at least one week following the surgery to check for any evidence of pain, and if required, will be given additional pain killers.

Disease model animals that develop movement problems that may impair their ability to access food and water will be given moistened food at floor level, and will weighed at least weekly (daily as required) to ensure they are remaining healthy.

Any animals undergoing heat stress will be monitored for body temperature every 15 minutes. If mice show evidence of hyperactivity during this time, this will be increased to every 2 minutes, and if temperature shows any evidence of spiking, the heat exposure will be immediately stopped.

Any animals undergoing frequent handling as a part of the experiment will be handled regularly prior to the start of the experiment, to ensure that this process does not induce unnecessary anxiety or stress to the mouse. In addition, all mice will receive appropriate training for all behavioural tasks prior to the onset of experiments, both to minimise anxiety and stress, and also to improve outcomes, which should ensure consistent data from all mice, and hence allow the use of the smallest number of tests and animals.

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

All work will be conducted following the general principals of the ARRIVE guidelines.

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

I will regularly check the NC3Rs website for updates, as well as liaising with our NTCOs and AWERB committee for any advances that might be relevant to this work. As far as possible, any relevant changes will be implemented immediately for all new experiments, and consideration will also be given to implementing changes to ongoing experiments, provided any such change is not expected to have an impact on the animals such that it may alter research outcomes.

29. Neurovascular breakdown in aging and disease and the development of vascular based 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

Neurovascular coupling, Neurodegeneration, Epilepsy, Alzheimer's Disease, Cerebral blood flow

Animal types	Life stages
Mice	Juvenile, Adult, Aged animal
Rats	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?

Neurovascular coupling is a mechanism that regulates blood flow in the brain. It is thought a breakdown in neurovascular coupling may be an important factor in disease and ageing, particularly dementia. This project licence will investigate how neurovascular coupling is operating in healthy brain and how it goes wrong in disease. We will also develop new treatments to change baseline blood flow and/or neuronal activity to assess whether this slows down or stops 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?

Currently there are very few treatments available to slow down or stop dementia. Our working hypothesis is that a decrease in blood flow to the brain is a major factor in the progression of dementia. Our research will understand precisely how this is happening and develop novel treatments to restore blood flow to normal levels.

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

There will be a number of benefits seen at the end of this project :

- All research will be written up in leading neuroscience journals and the data and analysis methods freely available as part of our funders' and the laboratories commitment to open and transparent research.

- We will further understand how neurovascular coupling works in the healthy brain and how it goes wrong in disease. A basic understanding of how neurovascular coupling works is still needed if we are ever going to development treatments to restore its function in disease.

- We will test novel vascular based therapies for brain disease and if successful seek to translate these into clinical trials.

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

This is discovery research in the short term. We are using animal models of human disease to fundamentally understand how the disease progresses from a neurovascular coupling viewpoint. By the end of this project licence we will have identified cellular targets for therapy and tested novel cerebrovascular therapies in our animal models. We will have assessed whether these therapies have had any impact on disease progression. Negative findings will be disseminated equally alongside any interventions that have potential benefit. This will enable other laboratories to understand which potential treatments to continue researching. The overall goal of this research is to develop therapies to slow brain disease in humans and by the end of this project license the expectation is that any treatments we develop that have a disease modifying effect will be taken forward to clinical trials.

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

The aim of our research team is to publish all of our experimental work including negative results. We are committed to open research in terms of making all of our data available to the scientific community upon publication. As a research group we have contributed several major review articles in our subject area to generate discussion and potential future directions for our research. Currently we collaborate with several research groups both within the UK and with other national and international leading research institutions. We also actively participate with activities to describe our research to the wider public especially charitable organisations.

Species and numbers of animals expected to be used

- Mice: 7000

- 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.

This project will use young, adult and aged rats and mice.

A key aspect of this project is to assess how neurovascular coupling is changing in health, disease and ageing. Therefore, we need to perform experiments across the animals lifespan. We chose the rodent model as we have 25 years experimental experience and it is the lowest form of sentient life that have a comparable brain and circulatory system to that of humans.

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

We will use wild type control animals and Genetically Altered animals for this project. The GA animals either will have a disease (e.g. Alzheimer's), bred with proteins called opsins in specific brain cells that we can then stimulate using light with optogenetics or bred with fluorescent molecules (GCAMP6) in brain cells that emit light following stimulation. For the animals with disease, we want to assess whether neurovascular coupling is breaking down in disease and develop new treatments to test on them. For the optogenetics, we want to assess how a specific cell, for example an interneuron is involved in regulating blood flow in the brain and to potentially use stimulation of that cell in the treatment of disease. For the GCAMP6 animals it allows the measurement of brain activity without the need to put an invasive electrode in which represents an important refinement in our methods.

Generally, acute non-recovery experiments will be used in the first instance to assess basic response and control stimulations. Then we will use chronic recovery experiments in separate animals that last in general for several months. These experiments may include a period of training or pre-treatment for the animal, followed by surgery to implant an imaging chamber to the skull. We have been doing these surgeries for a number of years and the animals recover quickly. Then the animal will enter a phase of longitudinal recording from the brain, either with recovery anaesthesia or awake. Followed usually by a final acute non-recovery experiment before the animal is killed by schedule 1 method or transcranial perfusion to collect the brain for post mortem histology.

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

Some animals will undergo multiple surgical procedures (head implant and osmotic pump placement to deliver drugs) and anaesthetics. In general the animals recover quickly and well from both procedures and anaesthetised imaging studies. Analgesia will be routinely provided following surgery. One adverse effect of recovery surgery will be a small reduction in weight which tends to recover quickly. From our previous research there has been no evidence of any cumulative effect from repeated anaesthesia's. Animals used in awake imaging protocols habituate rapidly to the head restraint and we will use positive

reinforcement following training and experimental sessions. Some animals will have an altered diet (e.g. high fat western diet) for the duration of the study which may give the fur a greasy appearance. Many of the chronic experiments can last up to several 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)?

In all acute anaesthetised experiments the severity will be non-recovery. In our chronic experiments all animals that have cranial implants will be at a moderate severity. For mice the overall percentages of severities will be 70% mild, 15% moderate and 15% non-recovery. For rats 100% of the animals will be non-recovery experiments.

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?

To understand neurovascular coupling in the brain, it is necessary to generate relevant biological data. For our research we need an intact working central nervous system with a working bloods supply. The use of animals is therefore unavoidable. Rats and mice are the animal models of choice for research in this area due to the well-defined brain structure and function. That is, the sensory cortex of these animals functions in a way very similar to human brains. Our expertise in using these animal models for research has been developed over a 20-year period. We will also use specific mouse strains that reflect human disease such as Alzheimer's and they will provide important information about disease progression.

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

As we need a functioning brain with a circulatory system there are no non-animal alternative for this work. Non-living animal alternatives such as brain cell preparations, brain organoids and nonprotected species such as insects have been considered but critically none of them exhibit neurovascular coupling either through a lack of a circulatory system and/or all the cells of the neurovascular unit. In future artificial systems may exist for modelling of the responses we expect to see (called in silico medicine) but at present they are not available.

Why were they not suitable?

They cannot simulate the concerted action of millions of brain cells and a reactive vascular supply.

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 estimates are based on previous research and ongoing experimental work in the laboratory. In all experiments we routinely use statistical software and robust pilot data to calculate the numbers of animals need to reach appropriate statistical power in all our experiments. We can therefore achieve robust scientific results with appropriate group sizes. All our grant applications have specific sections dedicated to the statistical calculation of group sizes and we adhere to ARRIVE guidelines with respect to randomisation and blinding in our experimental approach and analysis. Our estimate of animals is the minimal number of animals needed to generate scientific data that is statistically robust.

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

Our group have used the NC3R's Experimental Design assistant and G-Power software to generate group size estimates need to achieve robust significant results with appropriate groups sizes with the fewest animals needed to achieve appropriate statistical power. A specific example of our experimental design is we have recently published where we used a 2-factor ANOVA repeated measure design to assess neurovascular breakdown in the J20 Alzheimer's mouse. As we were able to repeatedly measure the same mice over a period of 4 months (blood flow response to sensory stimulation) we were able to achieve robust results using only 10 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 liaise with our biological services unit to ensure all breeding protocols are efficient as possible and where possible all non-transgenic animals will be used as controls. All our group size estimates are based on robust pilot data. We are also actively collaborating with an international academic team who use computer modelling of our data to understand potential mechanisms to explain our results. This modelling work will allow a more targeted approach in our future work. Finally, we are one of the leading laboratories in using multiple techniques in the same animal simultaneously and over multiple recording sessions. This allows the collection of higher quality data from fewer 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.

Our approach will be to use non-recovery terminally anaesthetised animals in the first instance to refine experimental approaches. Then we will use chronic experiments where we can repeatedly take measurements from the same animal over many months. This reduces the number of animals needed compared to using multiple animals at separate time points. We carefully train all staff before they are allowed to perform experiments and animals are constantly monitored during studies to ensure suffering and stress are kept to an absolute minimum.

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

We have to perform longitudinal studies for this project as we need to assess disease progression as part of our studies and develop potential new therapies as part of the project. We cannot use less mature animals because neurovascular coupling isn't established in very young animals and our main disease of interest is Alzheimer's which only occurs in adult or old animals. In our opinion the rodent model is the least sentient animal we can use while still being able to compare to results seen in humans.

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

We already operate high standards in monitoring and post-operative care of our animals. We have daily contact with the Biological Services Unit and the named animal care and welfare officer to pick up on the earliest signs of potential distress in our animals.

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

We will keep up to date with the NC3R's ARRIVE guidelines and update members of the team in all aspects of best practice.

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

The project licence holder is a member both the University Animal and Welfare Ethical Review Body to keep up to date with new developments and best practice. A member of our research groups has held a grant from the NC3R's. We routinely have both within lab and cross lab research group meetings to disseminate best practice.

30. Drivers of cancer drug resistance

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, APOBEC, Drug resistance

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 cancers become resistant to treatment, to determine the role that a set of seven human genes (the APOBEC3 'A3' genes) plays in this process and to test whether blocking A3 function can improve therapeutic responses.

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?

If cancers are diagnosed at an early enough stage, they can be cured by surgery. Unfortunately however, many cancers have already spread beyond the tissue in which they first arose by the time they are diagnosed. These require chemotherapy and more recently, treatments referred to as targeted therapies and immunotherapies. These treatments often work well initially and patients become well again. However, it is sadly the case that often the cancer will recur after several years, months, or even weeks and at this point, the tumour cells can no longer be killed by the treatment that was previously effective. It is therefore an urgent priority that we understand how this phenomenon, often referred to as acquired drug resistance, occurs and how we can prevent it from happening.

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

The human A3 genes we study encode proteins that help to protect us from infection by many different viruses, including HIV and SARS-CoV2. They do this, at least in part, by attacking viral DNA or RNA and altering the genetic code that the virus needs to replicate. This protection appears to have come at a cost however, as there is strong evidence that many of the mutations found in the DNA of cancer cells are generated by off-target A3 activity. These mutations ('mistakes' in the genetic code) cause cancer and can enable cancer cells to become resistant to cancer drugs. Until now, it has not been possible to study this in mice, as they do not possess the human A3 genes that make mutations in cancer. By using a new mouse line that has all seven human A3 genes (generated and characterised under my current PPL), we will:

Demonstrate the effect of the human A3 genes on the ability of cancers to become resistant to a variety of cancer treatments that are of direct relevance to human patients.

Demonstrate the effect of the human A3 genes on the ability of the immune system to detect and respond to cancers.

Demonstrate whether inhibition of A3 gene activity can prevent or delay the development of drug resistance.

All these findings will be published in international peer-reviewed journals and presented at scientific conferences. They will also be shared with collaborators developing A3 inhibitors as cancer drugs. If successful, the studies conducted under this licence will directly contribute to the clinical testing of A3 inhibitors by the end of this project. If negative results are obtained, these will be published in peerreviewed, open access journals wherever possible.

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

Researchers in academia and industry that are developing A3 inhibitors as cancer drugs will directly benefit from the information we generate, as our studies will provide important proof-of-principle that the strategy of A3 inhibition could be used to prevent drug resistance in cancer patients. These benefits will begin to be realised within the first 2 years of the project.

Due to the fact that we are collaborating closely with researchers developing A3 inhibitors, we anticipate that if these inhibitors are able to prevent drug resistance in one or more of our tumour models, clinical trials will commence by the end of the project and therefore it is possible that cancer patients will begin to benefit within 5 years.

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

We will publish all results from this project in peer-reviewed journals as soon as possible (i.e. once we are sure our findings are reproducible and interpretable, and allowing for the protection of any intellectual property arising by patenting - particularly important for the A3 inhibitor work). We also have a policy of posting pre-prints of our work upon initial submission for peer review, which means our manuscripts are freely available to all to read and use under a CC-BY open-access licence (the least restricted form of open-access publishing, in which content may be freely reproduced and used for teaching or other purposes, so long as the original source is cited) several months prior to final publication.

We are collaborating closely with both a small start-up company in the UK developing A3 inhibitors and large pharmaceutical companies that want to understand how tumours

become resistant to their existing cancer drugs, and to drugs they are currently developing. This collaboration will greatly increase the impact of our work and will maximise the chances of patient benefit.

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.

We are using mice as they are a long-established and very useful model for studying cancer development in humans. Importantly, we can engineer tumours with the same mutations that are seen in human cancers and that are treated with specific drugs that work only in tumours with these mutations ('targeted therapies'). We also know that the immune system is a critical factor in determining how tumours respond to cancer treatment. Modelling this component necessitates the use of mice as opposed to alternatives such as frogs or fish. It is necessary to use adult mice, due to the way in which the tumours are induced at sites that are relevant to the human disease and due to the time it takes for tumours to develop, and for drug resistance to occur during treatment.

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

In a typical study, lung cancer development will be induced by the administration of viral particles directly to the upper airway / lungs, either by inhalation of a droplet through the nose (intranasal instillation), or through a tube into the windpipe (intratracheal administration) while the mice are under mild (isoflurane) anaesthesia (this procedure takes approximately 5 minutes / mouse). Tumour development will be monitored using non-invasive imaging (typically micro-CT scanning) under anaesthesiaat approximately 8 weeks post tumour induction and at regular intervals thereafter (typically once / month and no mouse will be CT-scanned more than 18 times). Mice will be treated with anti-cancer drugs known as targeted therapies, at doses that have been demonstrated to be safe, and these drugs will be administered either orally (oral gavage, maximum once daily) or by another suitable and approved route, most commonly intraperitoneal injection (maximum twice daily). These targeted therapies are much less toxic than conventional cancer chemotherapy drugs, as they specifically target proteins that become essential for the survival of cancer cells but not for healthy cells. The drugs that we will use primarily in this project have not displayed any significant toxicity in mice, even with daily dosing over periods of several months. Dosing may continue for up to one year and mice will be closely monitored for signs of drug toxicity and/or suffering due to lung cancer burden. In some cases, we will administer experimental drugs (A3 inhibitors) in combination with the anticancer drugs to test whether we can prevent tumours becoming drug-resistant. We will only use A3 inhibitors once we have received detailed information on their safe usage in mice and on any potential toxicities from our collaborators who are developing them. Animals reaching pre-defined humane endpoints, and any animal alive at 1 year after the initiation of treatment will be culled via an approved Schedule 1 method. Tumour induction will typically be conducted on mice between 8-12 weeks of age and tumours typically take between 8-20 weeks to grow to a sufficient size to commence treatment, depending on the model. Therefore, even those mice that receive treatment for the maximum of 12 months without experiencing tumour recurrence would typically be killed by the age of 20 months.

We have included an optional step to keep mice alive to 2 years of age, in case of delayed tumour development, rather than wasting animals in which tumours develop more slowly than expected.

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

Mice injected with a drug are expected to experience mild and short-lived pain at the time of injection but we don't anticipate any longer-term discomfort. Similarly mice receiving drug by oral gavage will experience short-term discomfort but no lasting harm/discomfort.

Mice in which lung tumour development is induced may experience symptoms related to reduced lung function, most commonly breathing difficulties and/or weight loss. Mice displaying such symptoms will be closely monitored and culled as soon as a humane endpoint is reached.

Mice approaching two years of age may experience symptoms related to ageing, and all mice over 15 months of age will be closely monitored for signs of any such symptoms.

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)?

Approx 90% of mice in this project will be used for tumour induction studies, and they may experience discomfort of moderate severity. No animals are expected to experience anything more than 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?

We need to understand how tumours develop and how our genes of interest contribute to this process in the context of a living organism. Factors such as inflammation, disturbed sleep cycles and obesity can all increase our risk of cancer development and it is impossible to model these in cells growing in culture. We also need to establish whether new drugs targeting the A3 genes are likely to be effective before they can be trialled in humans.

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

We have conducted extensive analysis of the A3 genes in human clinical samples using DNA sequencing and gene expression data from The Cancer Genome Atlas project and International Cancer Genome Consortium. We have also made extensive use of human cell culture models, which form the main focus of our current research and we will continue to use human cell cultures wherever possible. We have invested a lot of effort in generating A3 knockout and A3-tagged human lines. We have also been successful in

generating cell lines from the lung tumours that we have generated in these mice under our existing licence. Wherever possible we will use cells derived from our mice rather than the mice themselves.

Why were they not suitable?

Human cell cultures will continue to be our primary research tool and will inform our animal experiments. However, the key questions concerning the role of A3 genes in cancer development and progression are only addressable in an animal model. Furthermore, the development of A3 inhibitors as anti-cancer agents depends upon establishment of appropriate preclinical models for testing prior to trialling in human cancer patients.

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 planned a detailed series of mouse studies that we need to conduct over the entire period of the licence to achieve our objectives. For each experiment, we will use the minimum number of mice required to give us robust, meaningful data.

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

We've followed guidelines detailed in the 'The Designing of Animal Experiments: Reducing the use of animals in research through better experimental design' (Festing, Overend, Borja and Berdoy; 2nd Edition). We have also used NC3R's Experimental Design Assistant to help plan 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?

We will use pilot studies to help refine our estimates of expected effect sizes. We will collect multiple tissues from euthanised mice to learn as much information as possible per mouse. We will also derive and culture cells from mice and will use those for analysis and further experiments (e.g. drug treatments), rather than performing procedures on live mice wherever 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.

This project is entirely based on the use of adult mice, in which we will model tumour development and treatment. We will use non-invasive imaging to monitor lung tumour burden, so that experimental mice that are not being used for experiments designed to measure time-to-reach human endpoint (a surrogate for terminal tumour burden) can be killed prior to the onset of any clinical signs of harm. We have carefully chosen humane endpoints to ensure that mice experience no more than moderate severity.

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

We need to study tumour development and the genes in which we are interested act in humans over many years to generate the mutations in DNA that eventually cause cancer in adults. These genes are not relevant to childhood cancers, so we need to study tumour development in the adult mouse. While a number of cancer-relevant studies can be conducted in non-protected animals, such as nematodes or flies, modelling the complex interplay between A3 gene expression and inflammation and/or viral infection and cancer development requires a model as close to humans as possible, hence our use of mice. Furthermore, we anticipate using these mice as a preclinical model for A3-targeted drug development. Again it is essential to conduct such studies in an organism that best approximates the action of a potential drug in patients, while avoiding the use of (for example) non-human primates. Indeed, by generating the humanized A3 mouse model, we have enabled these studies to be conducted in mice, as opposed to in non-human primates, which are the only other species apart from humans to possess the 7 A3 genes.

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

All work will be conducted by trained personnel, who will be given further training in specific techniques where necessary. This licence contains only protocols categorised as mild or moderate and anaesthesia and/or analgesia are used wherever appropriate. Animals will be closely monitored during all procedures and where possible we will consider refining existing techniques or incorporating new methods to minimise any suffering to the animals.

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

Ullman-Cullere et al 1999 'A Rapid and Accurate Method for Assessing Health Status in Mice' Lab Animal Science; 49(3):319-323.

Wilkinson et al 2019 'Progressing the care, husbandry and management of ageing mice used in scientific studies' Laboratory Animals 54(3):1-14.

Turner et al 2011 'Administration of Substances to Laboratory Animals: Routes of Administration and Factors to Consider' J Am Assoc for Laboratory Animal Sci 50;600-613.

Workman et al 2010 'Guidance for the welfare and use of animals in cancer research' Br J Cancer 102;1555-157.

Diehl et al, 2001 'A good practice guide to the administration of substances and removal of blood, including routes and volumes' J. Appl. Toxicol. 21, 15–23.

'The Design of Animal Experiments: Reducing the use of animals in research through better experimental design' (Festing et al 2nd Ed).

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

Our NTCO disseminates any 3Rs training or events at our quarterly Facility User meetings.

I will also continue to read the latest relevant scientific literature, attend conferences and consult policies on animal research from relevant funding bodies (Cancer Research UK, UKRI, NC3R's and the NC3R's Oncology Network.

31. Epigenetic reprogramming influences on ageing and regeneration

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

Rejuvenation, Cancer, Longevity, Ageing, Epigenetics

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 our project is to understand the mechanisms involved in cellular rejuvenation and assess its potential as a 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?

Age significantly influences the onset of diseases and the healing of complex wounds. Current wound healing strategies, such as antibiotics and dressings, are primarily effective for minor injuries, highlighting the need to explore cellular rejuvenation for larger and more intricate wounds. In blood, the ageing process leads to an increased production of specific cell types, raising the risk of age-related diseases like leukemia and myeloproliferative neoplasms. These chronic conditions severely impact quality of life and lead to complications such as strokes and hemorrhages, with no known cure other than bone marrow transplantation. However, finding suitable donors is challenging, especially for older patients whose blood stem cells are less fit. Cellular rejuvenation presents the

possibility of generating healthy bone marrow from affected patients and rejuvenate aged blood cells, potentially improving transplant outcomes.

In the realm of immunotherapy, CAR T-cell therapy involves modifying a patient's T cells to target and attack cancer cells. The success of this treatment largely depends on the quality of the initial T cells; if they are already exhausted, the therapy may be less effective. Therefore, strategies that preserve early-stage cells and prevent exhaustion are crucial. Cellular rejuvenation could revitalize these cells, enhancing their effectiveness and improving therapeutic outcomes.

Beyond blood and immunotherapy, the potential applications of cellular rejuvenation extend to developing therapies that prevent a wide range of age-related diseases, offering a promising avenue for future medical advancements.

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

This project aims to comprehensively characterise the adverse effects of ageing on various tissues, including skin and blood. This characterisation will enable us to evaluate the effectiveness of our cellular rejuvenation strategy through a comparative analysis of aged and rejuvenated tissues. In the long term, our goal is to develop a cellular rejuvenation therapy suitable for clinical application.

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

The skin research will contribute to the advancement of therapies aimed at treating or alleviating symptoms associated with skin cancer, chronic wound healing, hair follicle loss, and loss of pigment in hair.

The blood research will contribute to the development of therapies for treating tumours and blood cancers, such as leukaemia, and improving the current success rate of bone marrow transplants.

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

We have established collaborations with various research groups, each focused on related fields. These collaborations are expected to accelerate our progress and enhance our comprehension of the mechanisms that govern cellular ageing. This, in turn, will contribute to the creation of more potent rejuvenation technologies.

The findings of this research will be shared through publication in peer-reviewed journals or through patenting, especially when commercial potential is identified. In both instances, the resulting papers and patents will be made publicly accessible, fostering interest, attracting funding, and propelling progress in the field of therapeutic rejuvenation.

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.

Mouse models have been extensively employed in immune and blood research, primarily due to their well-established suitability for such studies. The transplantation of blood stem cells is considered the gold standard method for assessing their stem cell function. Additionally, our research group delves into the intricate interactions among blood stem cells, tumours, and their niche, encompassing various cell types and structures such as bones, vessels, and nerves. The complexity of these interactions poses challenges for modelling them in vitro. Moreover, replicating blood flow and the nervous system using cell culture methods proves to be a formidable task.

Mice offer extensive opportunities for genetic manipulation crucial to our work. Access to primary and secondary lymphoid organs, along with peripheral tissues like the lung, is necessary for the proposed analysis, and such access is limited in human studies.

This project aims to identify positive therapeutic effects that rejuvenation may have on skin tissues in wound healing and on blood cells in bone marrow transplants. Juvenile mice will serve as positive controls to assess whether rejuvenation yields the desired positive impact. Aged mice will be employed, as rejuvenation therapies are anticipated to be most beneficial for human patients aged 60 and older. The relatively short natural lifespan of mice accelerates the study of the ageing process, expediting the development of therapies for human age-related diseases. Significantly, the ease with which mice can be genetically altered allows for the tracking of donor and host cells, facilitating the study of the effectiveness of blood stem cell engraftment — a key indicator for the successful outcome of bone marrow transplants.

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

In skin studies, animals typically undergo a surgical procedure under general anaesthesia, involving a skin punch not exceeding 10mm in diameter. We will evaluate the benefit of rejuvenation on the recovery process. Upon completion of the protocol, mice will be humanely killed and samples collected.

For blood studies, animals will be exposed to irradiation to deplete their current blood system, followed by injection with donor blood cells, commonly administered through a tail vein. Post-transplantation, blood samples will be collected from the animals to assess their recovery and response to rejuvenation. At the end of the protocol, mice will be humanely killed and samples collected.

In studies examining safety with respect to cancer, mice may be injected either subcutaneously (under the skin) or intravenously (tail vein) with cancer cells of interest. Our interests include whether our rejuvenation methods impact the tumour burden and how the CAR T cells react with the tumour cells. Therefore, the animals will be humanely killed while the tumours are still small.

These experiments will help determine the safety and efficacy of therapies which can be progressed to help human patients.

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

In most instances, animals are not expected to exhibit harmful phenotypes. Nonetheless, some animals may have an altered immune system, rendering them more susceptible to infection. Animals with modified immune status will be housed in a barrier environment, thereby minimising the likelihood of compromising their health.

Furthermore, aged mice often display grey fur and increased weight. Additionally, ageing animals may occasionally experience partial or complete blindness, primarily due to age-related vision loss and, less frequently, the development of cataracts or corneal dystrophy. They may also engage in scratching behaviour, resulting in minor superficial wounds. While the loss of vision is not anticipated to affect the mice's behaviour, if it is caused by a cloudy eye, it can lead to discomfort and, in the worst-case scenario, ulceration of the eye.

Animals that have undergone irradiation or treatment with specific drugs, such as tamoxifen, are more susceptible to weight loss. Therefore, these animals will be closely monitored and weighed regularly.

Some animals receiving subcutaneous (under the skin) injection of tumour cells may exhibit discomfort due to the tumour mass, which could impede normal movement.

Animals used in wound healing studies typically experience a rapid recovery from surgery. However, they may display signs of swelling, which typically resolves within 48 hours. The entire wound healing process naturally concludes within 14 days post-surgery without any intervention.

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)?

Model	Severity	Percentage
Mice	Mild	60%
Mice	Moderate	40%
Mice	Severe	0%

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?

As we investigate the interplay of bodily systems, it becomes essential to employ whole animals for determining these factors. Mice have long been a staple in laboratory studies, given that the outcomes often prove replicable in larger mammals and, eventually, humans. Attempting to replicate our experiments on small tissue samples is impractical, as it hampers our ability to measure broader microenvironment interactions, particularly those involving the nervous system and blood flow.

Whenever feasible, we resort to alternative methods such as in vitro cell cultures. In this approach, previously preserved cell lines are grown outside a living animal. These cultures

prove valuable for cultivating stem cells and simulating the microenvironment in short-term experiments. Nevertheless, the eventual transition to animal research is imperative to comprehensively study the interactive effects within the entire bodily environment.

Moreover, healthcare regulators mandate animal studies to establish the safety and efficacy of therapeutic procedures before their initial application in humans.

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

We have employed various non-animal alternatives to minimise animal usage to the greatest extent possible. Our approach involves utilising advanced 3D cell culture systems for researching mouse oesophageal tissue without causing harm to animals. Additionally, we employ cancerous cell lines in tissue culture to investigate the effects of our methods on cancer. The primary objective is to comprehensively comprehend the fundamental mechanisms of tissue rejuvenation. Animal usage is only contemplated when it is confirmed that such utilisation is essential to sustain valuable research avenues that cannot be pursued through non-animal systems.

Why were they not suitable?

Though our goal is to extract valuable data from non-animal systems, they fall short of replicating the intricate tissue environments like the bone marrow found in real animals. The latter comprise numerous cell types interacting to perform functions like wound healing. Consequently, there exists a limitation in our ability to fully showcase the safety and efficacy of a technique solely through tissue culture systems. To advance therapies to a point where tangible benefits for human patients can be demonstrated more effectively, the inclusion of animals in our research becomes necessary.

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 required number of animals based on the various experiments necessary to obtain statistically significant results, facilitating the efficient progression of therapies toward human studies. Drawing from our conducted pilot studies, we can strategically design experiments expected to produce valuable data and determine the optimal quantity that can be executed to high standards within a specified timeframe. Furthermore, we minimise the number of animals used by precisely aligning the breeding of mice with the experimental needs, implementing rigorous monitoring of colony size.

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

To minimise the utilisation of animals, our approach involves maximising the use of tissues from each individual animal and storing various samples suitable for diverse assays. Excess tissues will be made available to collaborators and other researchers upon request, contributing to a reduction in the overall number of mice required for research.

The entire program's animal usage will be streamlined through meticulous planning and scheduling of breeding and experiments, ensuring that the minimum necessary number of animals is employed to address research queries and conduct unbiased studies.

Moreover, in previous studies, we have adhered to the NC3Rs guidance and utilised the experimental design tool (https://www.nc3rs.org.uk/experimentaldesign-assistant-eda; https://nc3rs.org.uk/3rsadvice-project-licence-applicants-reduction). We have also considered the PREPARE guidelines, guiding our animal experiment planning to achieve meaningful results while minimising the animal count. These tools, along with insights gained from prior projects, will be instrumental in our ongoing commitment to reducing animal usage to the greatest extent possible in the proposed project.

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 employ standard and efficient breeding techniques to minimise the necessary number of animals bred for the eventual use of mice in our studies. Each protocol and experimental line will undergo pilot studies involving small animal numbers (around 7 to 10 according to statistical power calculations) to ascertain the likelihood of obtaining valuable data in larger studies and to avoid unnecessary animal use. For the majority of experiments, pilot studies have been previously conducted and will not be replicated in this licence; data from these earlier studies will guide the efficient attainment of significant results with the least possible number of animals.

Following the humane killing of animals used in experiments, we will systematically collect and store all available tissues to obviate the need for repeat experiments. Additionally, we may offer some of this tissue to other research groups, promoting widespread discovery and maximising the impact 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.

Using mice as a model system offers several well-established benefits, including a short breeding time, large litter numbers, a short life span, ease of genetic modification, and straightforward translation to human therapeutics. We specifically employ mice in our research because many desired genetic modifications have already been established in other labs, allowing for immediate comparability with published literature.

When utilising genetically-altered mice, our preference is to employ models that enable us to control the manifestation of disease or harmful phenotypes, thereby reducing the time and severity of such phenotypes. We closely monitor these mice throughout the experiments. The majority of the mice we use are not anticipated to exhibit adverse effects, and the procedures we perform are designed to be minimally invasive, with an emphasis on avoiding long-lasting harm.

In our wound healing experiments, we employ purpose-built skin punches designed to collect tissue quickly, creating up to two wounds per mouse. These resulting skin wounds are small, promoting rapid healing and minimising animal discomfort. We restrict the size of skin punches to a maximum of 10mm in diameter, and the procedure is exclusively performed under anaesthesia and analgesia to minimise pain for the animals. Additionally, aseptic bandaging may be applied to prevent wound deterioration while allowing the animals to move freely.

For experiments requiring the irradiation of mice to eliminate specific cells and alter blood cell composition, we adopt a strategy to mitigate suffering. This involves splitting the irradiation and coinjecting helper cells.

In the investigation of immune cell and tumour interactions, some mice will be subjected to the development of skin tumours. Close monitoring is implemented to minimise the duration of illness in these animals, and humane euthanasia is employed if the tumours exceed a size of 15mm.

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

Our research necessitates the use of mammalian animals to accurately emulate various aspects of human biology, such as the skin and immune system. Mice offer the advantage of easy genetic modification, and their well-studied disease states enable experiments to generate valuable data more rapidly and efficiently compared to other animal models.

Our experiments necessitate the use of bone marrow stem cell transplantation which involves the animals being irradiated at the start, to deplete their existing blood system. One of our readouts includes engraftment efficiency. Unfortunately such experiments are not possible in less sentient animals such as zebrafish or drosophila.

In humans, the processes of wound healing and immune function undergo significant changes with age. Therefore, it is crucial to investigate therapies across different stages of mammalian lifespans, necessitating the use of live organisms. Given that cellular therapies for diseases require days to weeks to take effect, animals must be kept alive during these procedures.

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

In studies on wound healing, the size of skin punches will be limited to a maximum diameter of 10 mm, with a maximum of two punches per mouse. The procedure will be exclusively conducted under anaesthesia and analgesia to minimise discomfort for the animals. Additionally, aseptic bandaging may be applied to prevent wound deterioration while allowing the animals to move freely. Post-procedure, mice will be monitored for signs of pain, and analgesia will be administered as needed.

To explore the potential of cellular rejuvenation in enhancing the long-term survival and engraftment of blood stem cells, animals will undergo irradiation at the beginning of the protocol to deplete their existing blood system. Subsequently, they will receive injections of donor blood cells. The irradiation dose will be administered in a split dose to mitigate the potential risk of severe tissue damage from the conditioning regimen.

In ageing studies, endpoints such as behaviour, body weight, appearance, and biochemical markers will be used to assess markers of ageing. This approach ensures that animals are not subjected to prolonged pain until death from old age. These markers will

be continuously monitored to ensure the well-being of mice, and humane culling will be implemented to prevent unnecessary suffering if necessary.

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

We will adhere to guidelines pertaining to record-keeping, surgery, education, training, and reporting of experimental results. Additionally, we commit to following the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines, which encompass aspects such as study design, randomization, bias prevention, and statistical analysis of results.

For all procedures and animal care, we will strictly adhere to the guidance notes and webinars provided by the National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs) (https://www.nc3rs.org.uk/welfare-assessment) and those from the Jackson Laboratories (https://www.jax.org/news-and-insights/jaxblog/2016/march/experimental-design-top-four-strategiesfor-reproducible-mouse-research).

In the context of cancer studies, we will refer to the guidelines outlined by Workman et al. in

"Guidelines for the welfare and use of animals in cancer research" (2010, Br J Cancer 102(11): 15551577. doi: 10.1038/sj.bjc.6605642).

For management of aged mice, we will refer to guidelines outlined by Wilkinson et al. in “Progressing the care, husbandry and management of ageing mice used in scientific studies” (2020, Sage Journals 54 (3): 225-238. doi: 10.1177/0023677219865291).

By consistently following and consulting these guidelines, we aim to contribute to a culture of transparent, efficient, useful, and reproducible research. This commitment is essential in minimising animal suffering and ensuring that any such suffering is only justified in instances where it is necessary for significant contributions to medicine and science.

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

We will consistently check the NC3Rs website to stay updated on the latest information and advice regarding the refinement of our procedures and the minimization of suffering. Additionally, we will refer to the RSPCA website (https://science.rspcs.org.uk/sciencegroup/researchanimals) for additional information. Our mice will be managed by highly experienced technicians in our Institute who employ the tunnel handling method. These experts in animal experimentation will be consulted regularly to ensure the strictest standards in mouse handling are consistently followed.

32. Fish health and immune function

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 animalsreared for agricultural purposes

Key words

Fish Health, immune, nutrition, vaccination, development

Animal types	Life stages
Salmon (Salmo salar)	Juvenile
Rainbow Trout (Oncorhynchus mykiss)	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 is to improve the basic knowledge of the fish immune system. The fish immune system is impacted by pathogen exposure and infection, vaccination, nutrition, developmental stage and environmental stressors.

Advancing our understanding of how the immune system functions is of great importance to improve the wellbeing of the fish and improve production of fish as a sustainable human food source.

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 key benefits include:

Basic knowledge of the fish immune system enables us to understand how fish respond correctly to infection and immunological stressors. Immunological stressors could include non infectious agents such as vaccines, new dietary components, or naturally occurring agents such as micro jellyfish or plankton. This research will give new information on the evolution of the immune system and will help to understand immune responses in higher vertebrates.

Understanding fish disease responses and maintaining healthy stocks is central to aquaculture that provides healthy proteins and oils for human consumption. Globally aquaculture is the fastest growing food production sector and in the UK is worth >£1.5 billion. To ensure high welfare standards and high-quality food stuffs research into fish health and vaccination is essential. Part of health management is the use of vaccines for protection against key pathogens. There are still many diseases requiring new vaccines, research is required to design and test these newly developed vaccines. Additionally, methods to assess their effectiveness using minimal numbers of animals are crucial, contributing to the principles of Reduction, Refinement, and Replacement in animal testing.

The relationship between nutrition and health is complex, but in all animals it is well-established that feeds impact health. The nutrition / health relationship is important as more sustainable diets (based on non-fish sources) are continually being developed. This research will help explain the health benefits / impacts of different nutritional regimes and design of diets in the future.

Many fish are farmed across many continents, ranging from freshwater, brackish water and full saltwater environments, where they are exposed to natural environmental stressors that can affect their health. Methods to mitigate these occurrences and develop early health indicators are highly important for fish health management.

Finally, during development, capacity of fish to defend themselves from pathogens varies, especially during early life stages and also in Atlantic salmon when they migrate from freshwater to saltwater. In salmon when the freshwater stage (parr) changes to a salt water tolerant salmon (smolt), many hormones influence the fishs' physiology and the immune system is suppressed showing the reduced ability to fight off pathogens. This project will address how the immune system is regulated at sensitive periods to improve fish welfare and survival following transfer to seawater in wild and farmed fish.

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

Basic science:

Evolutionarily, fish are a highly diverse group of vertebrates and are the first animals to have an adaptive immune system. Fish live in highly variable environments and need to be able to exist in areas where they are under continuous threat from infection. Much of the work undertaken will be of basic science knowledge, relating to immune function and disease resistance, nutritional impacts on health and also how different development stages (such as smolting in Atlantic salmon) impact health and disease resistance. All the work will be done on salmonid fish, specifically rainbow trout and Atlantic salmon, the two major aquaculture species in the UK.

There will also be outputs generated for the industry which will include improved vaccines and biomarkers for fish health that are mentioned below.

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

Translation to industrial sectors:

The major goal of this Project Licence is to improve health and welfare of farmed fish and the research is undertaken in a knowledge transfer approach with industrial partners. Understanding basic immune function is a prerequisite for studying further challenges such as vaccination, nutrition and life history events. The aquaculture industry is worth over £1.5 Billion to the UK economy and is likely to continue to grow with stagnation of wild fisheries, in fact in a global context, over 50% of fish and shellfish consumed by humans is now derived from aquaculture. Improvements in health and performance of the farmed fish will ensure continued growth of the industry with better welfare for the fish.

Wild fish interests

Understanding immune function and its relation to fish physiology is also important for understanding wild fish biology. With the current poor survival of wild salmon, interactions with farmed fish and human induced changes to the environment that could impact on fish physiology and health as well as immune parameters need to be better understood.

Outputs and beneficiaries

Basic science will focus on the interaction of physiological changes and immune function in

ectothermic (cold blooded) fish. This work will be published in peer reviewed international journals with a major focus on academics, applied research and development (fish health, fish nutrition and fish production). The outputs will also be disseminated at international conferences which can vary in attendance from basic science to industrial focussed- such as World Aquaculture and European Aquaculture Society meetings. The timeline for basic science output is incremental and continuous.

During the research with industry partners, molecular tools will be developed- such as biomarkers, diagnostics, and vaccines for improved health assessment and immunological competence. These tools can be taken by industrial partners and used in improvement of health management. Similarly industrial production protocols for rearing fish can be implemented in aquaculture to improve the health and welfare of the animals. This has clear impacts on the economics of the industry and UK competitiveness. When our research is disseminated to public it should increase awareness of aquaculture practice and potentially increase fish consumption by human consumer.

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

The main outputs are in peer reviewed scientific publications and presentations at national and international conferences. The research team also works closely with industrial partners associated with aquaculture, these include nutrition companies, vaccination companies, breeding companies and production companies. The research outputs (both successful and unsuccessful) are also shared directly with the companies when they are involved in the projects. Dissemination of some projects is also through trade magazines.

Species and numbers of animals expected to be used

- Salmon (Salmo salar): 5000
- Rainbow Trout (Oncorhynchus mykiss): 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.

The project will focus on Atlantic salmon and rainbow trout as the two major aquaculture species in UK. The fish will be sexually immature fish from first feeding stage to when they would be used for commercial harvest, which is before final sexual maturation. Atlantic salmon undergo a “parr-smolt transformation” when they change from freshwater resident parr to marine stage tolerant smolts. This is a critical stage for heath and immunological competence and this stage is of great scientific interest. **Typically, what will be done to an animal used in your project?**

Fish will be weighed and length taken, and some fish may have blood taken on one or more occasions, both these will be carried out under anaesthesia. During protocols fish may be individually marked by inserting PIT tag that involves minor surgery under anaesthetic. Other specific examples of what might be done on the project are given below.

To assess immunological and health impacts from functional feeds fish will be fed experimental diets, usually these will be nutritionally complete. During feeding trials fish will be measured and weighed under anaesthesia. The diets are often termed functional feeds and can improve performance of the fish health. Diets may also contain new raw materials and our interest is in how they affect fish health and immune function. At the end of the feeding trial an immune or disease challenge may be carried out to examine how the fish on different diets respond.

To examine immunological responses to stimulants, these will be administered orally in the feed or by injection mainly into the peritoneal cavity or muscle, or by immersion or direct application to mucosal surface. For pathogen challenge (disease infection) the same methods will be used as discussed above with the inclusion of natural exposure on commercial fish farms. Cohabitation will also be used where a small number of heavily infected fish are kept with the fish to be challenged mimicking a natural route of infection. All procedures other than feeding, immersion and cohabitation are performed under anaesthesia. Under most circumstances anaesthesia will not be carried out more than once in 14 days and total number of time fish are anesthetised will be monitored during a trial. Vaccination trials will involve vaccine being administered by one of the methods described above, fish will then be kept for approximately 8 weeks until immune protection develops. Following this, a disease challenge will take place as described above, fish will be monitored to determine level of prevention of infection.

For natural exposure of rainbow trout on a commercial flow through fish farm, immunological development will be monitored by blood sampling to examine antibodies to naturally occurring pathogens in the environment. These fish will be maintained at POLE. The fish will be PIT tagged and blood collected every two months during a production cycle. This work will allow for assessment of potential therapies and future vaccine design.

During development, fish are kept on different photoperiod regimes in preparation of seawater transfer (smoltification) and also to increase feeding by increasing day length. During smolting process the immune system is known to be suppressed. We will alter photoperiod during developmental stages in line with commercial practice and examine

how immune function is altered. The impact of photoperiod will be examined in terms of immune response and seawater fish health.

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

During immune stimulation, fish can generate a proinflammatory or antiviral response. This is normally a short term response and experience shows this does not last more than 48 hours.

During disease infection trials, the effects on the fish will be disease-specific. Bacterial infections will cause changes in behaviour and the fish may lack swimming control and become less responsive to stimulation. Some bacterial diseases such as furunculosis may result in external lesions. Viral pathogens might not cause external changes, but there may be lack of swimming control. Some viruses, only cause changes to internal organs at a slow rate and may result in reduced growth rate; others act very quickly and can present changes to behaviour within one day.

When pathogens are used for disease challenge, following feeding trial, or under differing environmental conditions or vaccination trial, fish are strictly monitored to ensure minimal suffering and fish are humanely killed if they deviate from expected behaviour. For disease challenges aiming to evaluate resistance to disease, these can last up to two weeks, as fish may only reach a humane end point may only start to occur following one week depending on pathogen. In some disease models, fish do not reach a humane end point, and the output of infection response is followed by humanly killing the fish at a predetermined time point, followed by analysis of clearance of pathogen by molecular methods.

Feeding trials would typically last 10 weeks and these may be followed by immunological experiment or disease challenge. Feeding trials with functional feeds are expected to only have mild effects, but new raw materials may cause mild inflammation in the intestine, which may result in reduced feed intake and weight loss on occasion.

Changing environmental production protocols may include changes in photoperiod (day length) that are routinely carried out in industry.

During all procedures monitoring of fish health welfare is important. Monitoring forms for different procedures are developed and the period between monitoring varies depending on requirement. Trained personnel carry out the fish monitoring who can recognise humane end points or other welfare indicators. This varies from monitoring several times per day during peak response to pathogen, to minimum of once every 24h on mild procedure.

In the experimental fish, lack of swimming control and external lesions may cause mild to moderate distress and these can be used as indicators for 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)?

For both species the overall severity will be 50% moderate, this will include use of PIT tags when required and response to pathogens and immune stimulants. When a pathogen challenge or immune stimulation is occurring there is regular monitoring to ensure

suffering does not exceed moderate. 50% of fish will experience mild suffering as this will where suffering is limited is limited injection and exposure to 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?

For feeding, vaccination, immune response and the production-related protocols cell culture cannot be used. We use both cell lines and primary cell culture where possible, but whole animal use is essential as cell lines do not allow for complex immunological interactions between cell types, organs and pathogens.

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

We regularly use primary cell cultures derived from healthy animals following a humane killing method. These cells can be from tissues such as head kidney (rich in macrophages and T cells). Spleen is also an immune cell rich organ, and we also use muscle cells as these can be key indicators of protein reallocation under inflammatory stimuli. We also have a panel of fish cell lines from Atlantic salmon and rainbow trout. These can be of great use, especially to help understand cellular immune responses, and how cells respond to pathogens. We are continually researching development for other non-invasive methods of fish health assessment.

Why were they not suitable?

Cell lines and primary cell cultures cannot represent the integrated immune response of an animal. They do have excellent uses but in vivo work is essential.

We anticipate during the lifetime of this project that more cell lines will be developed to replace some of the whole animal research when this is appropriate.

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 total 9,000 fish are estimated to be used. Fish require to be held at a minimum density, otherwise behavioural hierarchies become established that result in stress and poor welfare of fish. The biological units during production protocols or diet trials are at the tank level, rather than individual. A typical feeding trial followed by a pathogen challenge might require 4 diets, with triplicate tanks per diet and 50 fish per tank. Further sampling over the time course, with 30 infected and 30 non-infected fish, would be sufficient for a pathogen trial. This relates to a total of over 200 animals involved in the trial. A vaccination

trial with appropriate controls may require up to 300 fish, which will include post vaccine sampling to determine immunological responses.

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

The minimum number of animals will always be used to achieve robust statistical analysis. The numbers will vary depending on the experiment being performed but experimental design will be in consultation with a departmental statistician, power analysis will be used to determine number of animals.

Measures will be taken to avoid duplication of experiments. The exception will be to validate a study under our own conditions as these may change with factors such as strain of fish, diet and environmental conditions. Measures taken to avoid unjustified duplication of procedures will include close monitoring of literature, conference attendance, and networking within the field.

The numbers of fish per tank will ensure that no strong social structures and excessive behaviour develops between individuals. To ensure this a minimum of 15 fish should be maintained per tank (of 250 L), but this number may vary depending on the size of the fish, in consultation with NACWO and NVS.

During experimental design we have used the ten ARRIVE (Animal Research: Reporting of In Vivo Experiments) recommendations, which include sample size to ensure robust analysis and randomization to avoid technical artifacts. PREPARE (guidelines for planning animal research and testing) are also adhered to, which include preparation of animals, dialogue with other scientists to ensure most up to date knowledge and ensuring facilities are satisfactory for experiments.

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 where we can. For example, if we have a new immunostimulant or pathogen model we will carry out preliminary experiment before a large-scale experiment. The pilot study will still need sufficient replicates to give confidence that the new methods work. These trials are often written up as publications or reports in their own right.

When an experiment is carried out, we make maximal use of the tissues, or even harvest additional tissues that can be used for other analysis and projects. At the trial design stage my research group (and external collaborators) can contribute to ensure maximal use of material and information is gathered.

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 work will be carried out using farmed strains of fish which are used to living in a manmade environment. The choice of which species (Atlantic salmon or rainbow trout) to be used will depend on the research question that is being addressed. Different species have different nutritional requirements, rainbow trout are normally retained in freshwater, whereas Atlantic salmon live in both fresh and saltwater depending on life stage. Experimental diets will be customized based on specific objectives and species requirement. Pathogens are often species specific and fish of different stages may be more or less susceptible to them. Pathogens will include bacterial pathogens, viral pathogens and parasites. Pathogen exposures will be for different times as infection dynamics can be highly variable. For example some bacterial infections may require a two week infection trial whereas slow developing parasites may require a period of months.

During a trial the fish will be monitored and to avoid unnecessary suffering. These observations will include abnormal swimming behaviour, or other external features such as lesions on the skin. We will define humane end points, which is when the animal will be humanely killed to avoid further suffering.

The viral model to be used is Salmon Alpha Virus (SAV), this virus causes damage to pancreas and heart. In our experimental model the assay for resistance to SAV is to assess viral load in tissues at time points after infection. Peak viral load is observed at around 2 weeks following which the fish clear the virus. Histopathology can also be used for more detailed assessment. Generally SAV does not results in mortality.

Bacterial pathogens to be used for both Atlantic salmon and rainbow trout are *Aeromonas salmonicida* (causative agent for furunculosis) and *Yersinia ruckeri*. These bacteria can result in mortality which normally peaks at around 7 days post infection. Monitoring for humane endpoints is carried out.

Oomycete pathogens would include Saprolegnia parasitica, which is commonly known as water mould. This appears as a slow growing fungal infection on the skin and fins. If left, fish succumb to secondary bacterial infections, but during a trial monitoring for humane endpoints is carried out.

This information will ensure the experimental procedures result in less suffering and distress of animals.

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

Fish are described as lower vertebrates, and for protected species under licence it is not possible to reduce to less sentient animals. Invertebrates are not an option as fish are the earliest animals that have an acquired immune system. Invertebrates do have an immune system, but the cell types and signalling methods are very different to vertebrates.

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

Fish are usually brought into the facility as young as possible to reduce transport stress, and then reared to the size needed. Transport of the fish is compliant with our Code of Practice and the fish are allowed to acclimatize to the aquarium for several weeks. The fish that are exposed to pathogens following either a feeding trial or vaccination may become infected and some of these animals may die from the disease. During infections, regular monitoring of fish is carried out, and the monitoring can

vary depending on the type of pathogen (or immunological exposure) and its virulence. For example, a highly pathogenic strain of Aeromonas salmonicida will require monitoring of

up to every 4 hours during peak infection, which will ensure that any fish with signs of infection or abnormal behaviour will be removed from the tank and humanely killed by a S1K. The peak response to infection normally is 2-3 days post challenge for this example. This has been a refinement in the protocols in the last 3 years.

Refinements may include using analgesics or local anaesthetic where necessary. Our procedures do not induce direct pain. We continually enhance our monitoring of animals especially when under immune stimulation of disease challenge. We are generally not so interested in the more advanced disease stages of the pathogens we use. Hence, for each model/pathogen a monitoring sheet will be developed for relevant welfare and humane endpoint criteria. To ensure that these welfare and humane end point criteria are being met monitoring will be carried depending on the progression of disease development e.g. every 6 hours if required.

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

Smith AJ, Clutton RE, Lilley E, Hansen KEA, Brattelid T. PREPARE: guidelines for planning animal research and testing. Laboratory Animals. 2018;52(2):135-141. doi:10.1177/0023677217724823.

Our facility has also adopted the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments, https://arriveguidelines.org/)

I have also read the Norwegian version of NC3Rs that has excellent information. https://norecopa.no/prepare

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

I regularly attend workshops run by Animal Facilities. In recent years I have also taught on licencing courses especially on animal handling and 3Rs. I run the animal handling section for fish at aquarium facilities. In addition to this I continually try and find ways to reduce animal usage. An example of improved advances in 3Rs has been using repeat sampling from animals and also non-invasive sampling such as using swabs rather than using whole fish for gene expression analysis in mucosal tissues. There is also an emphasis on liaising with key personnel, including Named training officer (NTO), Named veterinary surgeon (NVS) and Named animal care and welfare officer (NACWO).

33. Tendon and Ligament Repair

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

Tendon repair, Ligament repair, Scaffolds, Fixation devices, Medical devices

Animal types	Life stages
Sheep	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 enable medical device companies to study the safety and efficacy of novel therapies such as soft tissue grafts, scaffolds or fixation devices aimed at repairing tendons and ligaments in 2 main clinical areas, the rotator cuff in the shoulder and the anterior cruciate ligament (ACL) in the knee.

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 rotator cuff is a group of muscles and tendons that surround the shoulder joint, keeping the head of the humerus (upper arm bone) firmly within the shallow socket of the shoulder. Rotator cuff injuries are common and increase with age. These injuries may occur earlier in people who repeatedly perform overhead motions and they are also common in athletes. Physical therapy exercises can improve flexibility and strength of the muscles surrounding the shoulder joint and for many people with rotator cuff problems, these exercises are all that's needed to manage their symptoms. Sometimes, however,

rotator cuff tears may occur from a single injury or from degenerative diseases and in these

circumstances surgery is often required involving the use of scaffolds or fixation devices. An estimated 450,000 rotator cuff repairs are performed in the United States per year costing up to $19,000 USD per procedure and over 9,000 are performed in the UK costing up to £8,000 GBP per procedure.

Knee ligaments connect the femur (thigh bone) to the tibia and fibula (the lower leg bones). They are classified into two main groups; collateral ligaments which connect the sides of the bones together and cruciate ligaments which provide fixation and stability within the joint itself. Sprained and torn ligaments are common especially among athletes. They may be mild requiring merely rest and simple treatment or they may be more severe, requiring surgery. More than 175,000 Anterior Cruciate

Ligament (ACL) reconstruction procedures are performed each year in the US alone costing up to $50,000 USD per procedure and an estimated 30,000 procedures in the UK, costing up to £8,500 GBP per procedure. ACL reconstruction aims to reinstate the functional stability of the knee; in turn, preventing further damage to the knee cartilage and reducing the risk of degenerative osteoarthritis.

Current products do not always facilitate the anticipated return to normal patient activities and some can fail sooner than expected resulting in the requirement of a second repair procedure. Therefore, the fixation devices and repair therapies anticipated to be evaluated within this licence will be being developed with the intention of improving tendon healing and soft tissue graft fixation to accelerate healing responses, patient rehabilitation and to facilitate the return to normal patient activities.

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

The fixation devices and repair therapies will be developed with the intention of improving tendon and ligament healing and soft tissue graft fixation to accelerate healing, patient rehabilitation and the return to normal patient activities. The benefits to patients should be a stronger repair, improved range of joint motion when compared with current therapies and longer lasting repairs reducing the need for revision surgeries.

It is expected that the data from successful studies (those showing no adverse effects as a result of the novel materials and/or those showing improved tendon or ligament repair) will be submitted to the relevant regulatory authorities for approval and launch of these new products.

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

It is anticipated that members of the human population requiring rotator cuff tendon or knee ligament surgery will benefit. These patients' everyday activities will have likely been impacted by a traumatic event or through degeneration of the tendon or ligament. New repair therapies are likely to promote faster patient rehabilitation and better healing than current therapies and the resultant repair is expected to last longer. Longer lasting therapies will reduce the need for revision surgeries.

The surgical implantation of new repair therapies could be simpler through the implementation of improved surgical instrumentation and techniques as well as being more robust which will benefit surgeons.

Both of these benefits will in turn reduce the cost burden on healthcare providers.

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

The offering of validated tendon and ligament repair models as a service means that numerous medical device companies will be able to evaluate their products in these models. Where confidentiality is not breached data will be shared across organisations and where possible, publications of the work conducted under this licence will be considered.

Species and numbers of animals expected to be used

- Sheep: 900

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 adult sheep is our large animal model of choice for tendon and ligament repair studies as the anatomy and the size of the structures to be repaired are both similar to humans. In this way clinical implants i.e. those designed for use in humans can be evaluated without the need to scale up or down making the results much more likely to be accepted by a Regulatory Authority. In addition there is an adequate supply of suitably sized sheep in the UK (ideally aged between 2-5 years with a weight range between 60-100kg) and the joints are of a size that is suitable for mechanical testing and histological analysis which are the endpoint measures that will be used to determine success.

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

Sheep will be acclimatised to the facility and handling procedures prior to use.

Blood may be taken according to general principles on blood sampling on more than one occasion.

On the day of surgery they will receive a pre-medication containing an analgesic (painkiller) and will then be anaesthetised for the surgical procedure.

The surgical procedure will be performed aseptically (in a sterile manner that is free from harmful bacteria and microorganisms) and will involve the implantation of scaffolds, grafts or fixation devices to repair or fix surgically created injuries in knee ligaments or shoulder tendons. Surgical sites will be closed and the sheep will be recovered from the anaesthetic.

A typical procedure will take approximately 1 to 2 hours from anaesthetic induction to wound closure.

A cast or splint may be used for up to three weeks following the surgical procedure to immobilise and/or unload the joint to protect the surgical site and prevent initial dislodgement of the repair therapy (scaffold, graft or fixation device).

Post surgery analgesics (painkillers) will be used as required.

Following recovery, images such as x-rays may be taken to assess implant fixation and/or healing. An additional anesthetic will be required each time images are taken, so because of this they will be taken no less than 2 weeks apart.

At the end of the procedure sheep will be humanely euthanised and the implant/host tissue construct will be removed for testing and analysis.

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

It is expected that there will be a degree of post-operative discomfort and lameness which will be controlled by analgesics. This isn't expected to last longer than 24-72 hrs following surgery.

Sheep will be single housed during the immediate post-operative period. This is to prevent injury before the sheep have fully recovered but as they are a herding animal this could cause some distress. To minimise this distress a line of site will be provided to adjacent pen mates and group housing will normally be re-introduced 24-72 hrs following surgery. Re-introduction to group housing is expected to be without incident.

A cast or splint may be used for up to three weeks following the surgical procedure to immobilise and/or unload the joint to protect the surgical site. It sometimes takes a few hours (<24hrs) for the sheep to get used to these immobilisation devices but they are typically well tolerated and the sheep will be closely monitored during their 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)?

All animals are expected to experience a moderate severity 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?

Tendon and Ligament repair is a complex process involving cellular repair mechanisms and inflammation. In-vitro (lab based) cell culture studies cannot replicate the in-vivo (in a living body) loading, physiological and anatomical conditions required to demonstrate the safety and efficacy of novel repair therapies, therefore animal studies are necessary in their development.

In addition, the endpoint measures required to study the strength of repair and the tissue/cell types making up that repair are biomechanical and histological, both requiring the use of living tissue of a size and structure appropriate to the intended clinical environment.

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

In-vitro cell culture studies involving the use of synthetic bone scaffolds either unloaded or under some load to try to emulate the clinical environment in which the final products will be used.

Why were they not suitable?

In-vitro cell culture studies are useful as a screening method to assess the effects of novel materials on the viability of cells. These types of studies will be used to screen out any potentially harmful structures or materials before healing/repair is studied.

However, in-vitro cell culture studies cannot replicate the in-vivo loading, physiological and anatomical conditions required to demonstrate the safety and efficacy of novel repair therapies, therefore animal studies are necessary in their development.

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 total number of animals has been estimated based on typical study sizes and the expected numbers of studies required for the duration of the project. Power calculations will be used to determine the number of animals required for each study and this will be dependent on the specific study objectives.

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

As this is a service licence the experimental design phase for each required study has not yet happened.

When it does FRAME and NC3Rs guidance will be followed regarding reduction opportunities and the NC3Rs Experimental Design Tool (EDT) will be used where appropriate to inform the design of studies. Statisticians will be consulted in the planning stages of in-vivo studies to determine the appropriate study design, number of groups and number of animals required. Studies will typically be designed to 80% power, although this could differ, and could be designed, for example, as either superiority or noninferiority studies with appropriate limits depending on specific study objectives.

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

Historical data will be used to power studies where it exists. Otherwise pilot studies will be conducted to inform the design of subsequent pivotal studies. Control items will be used as appropriate so that the results from novel test items can be compared against known controls. Animal variability will be reduced as much as possible by the sourcing of a consistent and reproducible supply of sheep.

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.

Adult sheep (ideally aged between 2-5 years with a weight range between 60-100kg) will be used in this project as they have limbs which are of a sufficient size to study clinically relevant sized implants.

Several models are being proposed to evaluate the safety and efficacy of novel therapies such as soft tissue grafts, scaffolds or fixation devices aimed at repairing tendons and ligaments in 2 main clinical areas, the rotator cuff in the shoulder and the anterior cruciate ligament (ACL) in the knee.

The models proposed have been developed, validated and refined under three previous concurrent Project Licences over a period of 15 years.

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

Adult sheep have limbs which are of a sufficient size to study clinically relevant sized implants. Juvenile sheep would have smaller limbs which may not have a sufficiently similar structure in which to study the required repair therapies. These would also heal much quicker potentially masking any improvements provided by the novel therapies. Less sentient species are less suitable to meet the objectives of this work due to their size but the safety/biocompatibility of any novel materials may have been previously assessed in less sentient species as required by the relevant International Standards.

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

The models within this licence have been developed, validated and refined over the last 15 years however, opportunities for further refinement will always be considered. Guidance from institutes such as NC3Rs will be followed where appropriate.

Acclimatisation periods will be utilised and refinements in post-operative care and pain management will be utilised where these are proven to reduce harms to the animals.

Animals will be group housed where possible and where single housing is required following a surgical procedure, a line of sight to a pen mate will be provided by not having solid pen sides. Group housing will be reintroduced as soon as possible after a surgical procedure which is expected to be without incident. Good ventilation is essential when animals are housed indoors and when possible, animals will be moved out to pasture.

Environmental enrichment methods will be utilised. In sheep these are mainly limited to providing a variety of feed and feeding methods. In addition to feeding good quality hay/haylage ad-lib a scoop of pelleted diet can be added for variety, mineral licks and additional feeds may also be provided and supplements e.g. beet or other appropriate fruit/veg may be fed as a form of environmental enrichment. The method of feeding can also be regularly changed to add variety.

Surgical implantations will be practiced and refined in cadaver tissues as required.

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

LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017), NC3Rs, ARRIVE and PREPARE guidelines will be followed where appropriate.

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

Through general literature review, review of NC3Rs website, dialogue with the Named Information Officer and Named Training and Competency Officer as well as other establishments.

34. Investigating the pathogenesis and control of a zoonotic protozoan pathogen

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Protozoan parasite, Pathogenesis, Foodborne, Disease prevention

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 aim of this project is to investigate the pathogenesis of *Toxoplasma gondii*, an important pathogen of humans and animals, and assess methods for control and prevention 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?

Toxoplasma gondii is a parasite that impacts human and animal health worldwide. Infection in humans is usually very mild; however, there can be severe or life-threatening disease in immune compromised people or pregnant women. *Toxoplasma* also impacts the livestock sector where it can cause abortion in sheep and goats. Transmission of *T. gondii* through food is thought to be a major source of infection in people, particularly the consumption of raw or undercooked meat; however, there is a significant knowledge gap on the role of retail meat in the transmission of this parasite, and a lack of up-to-date

guidance on disease prevention. Variation in disease outcome (in humans and animals) is related to several factors, including the genetic diversity of the infecting strain of the parasite, with some strains causing more severe disease than others. It is, therefore, crucial to have an understanding of the virulence of different *T. gondii* strains, and to establish appropriate methods for the control and prevention of toxoplasmosis.

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

Outputs at the end of this project will include an increased understanding of the pathogenicity of different strains of *Toxoplasma gondii,* which could help predict disease outcome. We will be comparing strains of the parasite with the same genotype yet very different levels of virulence, which presents an excellent opportunity to identify fundamental differences between them and potentially identify novel markers for virulence. Results of the project will be presented at conferences, prepared for publication, and may form the basis of a future grant application.

A further aim of the project is to investigate suitable methods for killing *T. gondii* in meat products, so outputs of this work will include optimum cooking and freezing conditions to kill the parasite, which will help inform control and prevention guidelines for foodborne toxoplasmosis.

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

A better understanding of the impact of strain variation and *T. gondii* virulence will benefit those involved in diagnostics, screening, and disease prevention and control, in particular with at-risk groups of people. Outputs will also be of interest to those working on *T. gondii* host-pathogen interactions in both veterinary and human medicine. We plan to conduct cell culture-based assays alongside the proposed mouse work which will benefit those working on other pathogens and will help to promote the 3Rs principles in research to reduce, replace and refine work using animals.

The optimum cooking and freezing conditions to kill *T. gondii* tissue cysts in meat products will be of interest to public bodies such as Food Standards Scotland and the Food Standards Agency. Improved prevention and control guidelines for foodborne toxoplasmosis will benefit society by reducing disease, particularly in at-risk groups where infection can have serious consequences.

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

Results of the proposed work will be maximised and disseminated via presentations at international and national conferences where further collaborations will be sought out, and publication in openaccess peer-reviewed journals. Any relevant data will also be deposited in public repositories to aid other researchers working in the field.

Species and numbers of animals expected to be used

- Mice: 340

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, adult mice are the only accepted model for assessing virulence of *Toxoplasma gondii*. The mouse work we propose will be conducted in parallel with cell culture-based work using 3D "mini guts" (organoids), from mice and sheep, to allow a comparison of results and hopefully full replacement of mice (and other animals) in future research.

In order for us to investigate suitable cooking and freezing conditions to kill *T. gondii* in meat products, we must first produce meat products that contain the parasite. Rather than using tissues from experimentally infected food animals, we will spike meat products, purchased at the supermarket, with *T. gondii* tissue cysts (the stage usually found in infected meat). Currently, mice are the least sentient animal model available for producing *T. gondii* tissue cysts.

Once the spiked meat products have been cooked or frozen, we then need to determine if the *T. gondii* tissue cysts have been killed or if they are still viable and capable of causing infection. The only reliably sensitive method currently available to do this is the inoculation of mice. Recently, a cell culture-based method for determining parasite viability has been described, although it is not yet a widely accepted method and may not prove to be as sensitive as the mouse model. However, we will conduct the cell culture-based method alongside the mouse work to allow a comparison of results and hopefully full replacement of mice in future research.

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

For assessing the virulence of *T. gondii*, mice will be injected (once) in the abdomen with known numbers of parasites and monitored twice a day for 4 weeks. After this time, or if they reach a defined humane endpoint before this, they will be humanely killed. Tissues will be collected at post-mortem.

For the production of *T. gondii* tissue cysts for the meat-spiking experiments, mice will be injected (once) in the abdomen with known numbers of parasites and monitored twice daily for 8 weeks. After this time, or if they reach a defined humane endpoint before this, they will be humanely killed. Their brains will be removed at post-mortem for the isolation of tissue cysts.

For the determination of *T. gondii* viability in meat samples, mice will be inoculated (once) in the abdomen with a sample of homogenised meat and monitored twice daily for 4 weeks. After this time, or if they reach a defined humane endpoint before this, they will be humanely killed. Tissues will be collected at post-mortem.

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

Mice inoculated with *Toxoplasma gondii* alone or *T. gondii*-spiked meat products may be transiently affected by the initial inoculation. Signs may include short-lived fever and a ruffled coat. This is only expected to last for 24 hours. Thereafter, infection with the parasite may impact body condition (e.g. weight loss), coat condition (e.g. ruffled) and/or

demeanour (e.g. hunched posture) which will be monitored and scored twice daily. If a defined humane endpoint is reached, mice 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)?

Assessing *T. gondii* virulence: Approximately 54% of mice are expected to experience moderate severity and the remaining 46% are expected to experience mild severity.

Generating tissue cysts for meat-spiking experiments: We estimate a low proportion of mice (1030%) will experience moderate severity, and the remaining 70-90% will experience mild severity.

Determining *T. gondii* viability in meat products: We estimate that approximately 70% of mice will experience moderate severity following, and the remaining 30% 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?

At present, there are no reliable and sensitive cell culture-based alternatives for assessing the pathogenicity of *T. gondii* isolates or for determining the viability of *T. gondii* in meat products. For both aims, mice are currently the gold standard model. Virulence is assessed based on the number of mice requiring euthanasia (due to clinical signs) following infection with different doses of *T. gondii* - with virulent strains leading to 100% euthanasia, strains of intermediate virulence leading to 30-99% euthanasia, and non-virulent strains leading to less than 30% euthanasia. This standard model has the advantages of reproducibility, ease of inoculation, and accurate administration of challenge dose. For assessment of parasite viability in meat, mice are the only current model sensitive enough to detect the likely small numbers of *T. gondii* present in meat products as they are a natural host of the parasite.

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

Organoids are one of the most exciting stem cell technologies to be developed in recent years, recapitulating many of the physiologically relevant features of *in vivo* organs. They can be derived from primary tissue or pluripotent stem cells from a number of tissues, including the gut. Different organoid cultures have recently become available at our establishment, and we intend to conduct an assessment of *T. gondii* virulence (as

measured by infectivity and proliferation of parasites) in mouse and sheep intestinal organoids alongside the standard mouse virulence assay.

Recently, a paper has been published detailing a cell culture-based protocol for the determination of *T. gondii* viability (Opsteegh *et al*. (2020) Int J Parasitol. 50: 357). Although it is not as sensitive as the mouse bioassay (it can only reliably detect more than 1000 parasites, compared to 10 parasites detected in the mouse assay), we will utilise the cell culture method alongside the mouse bioassay to compare results.

In both instances, if *in vitro* results are comparable to those obtained *in vivo*, we hope to use the organoid and cell culture systems in any planned future work thus replacing the need for mice.

Why were they not suitable?

At present, the use of organoids has not been established or standardised for assessing *T. gondii* virulence in mice, and the cell culture protocol has yet to be demonstrated as sensitive as the mouse bioassay for assessing parasite viability. We will utilise both alternative methods in our planned work but cannot solely rely on these methods.

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 worked with a statistician at Biomathematics and Statistics Scotland (BioSS) to calculate the minimum number of animals required for experiments whilst maintaining statistical power. The calculations were based on estimates and results from previous experiments we have conducted at the Establishment, or from published literature.

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

I am aware of the PREPARE and ARRIVE 2.0 guidelines, and will utilise these when planning my experiments to ensure I keep the number of animals to a minimum. I am the secretary of the Moredun 3Rs Committee and have knowledge of the various online resources and tools for planning animal experiments, such as the NC3R's Experimental Design Assistant, which I will use to design my experiments. I also consulted closely with a statistician to ensure the minimum number of animals are used whilst maintaining statistical power.

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 maximum use of the mice, we will harvest as many tissues as possible at post-mortem following completion of the experiments and freeze them at minus 20. These tissues could be used for future further analysis not currently planned in the current experiments, and some tissues can be used as a source of *Toxoplasma gondii* DNA for controls for molecular methods. They can also be made available to other researchers. Tissues from negative control mice can be shared with the organoid group for the establishment of different murine organoids.

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 mice to assess the pathogenicity of *T. gondii* strains and the viability of *T. gondii* in meat products. To ensure we cause the least pain, suffering, distress or lasting harm to the animals, we will monitor them twice a day following infection with the parasite and use a scoring system to assess their demeanour and coat condition. A humane endpoint will be pre-defined and if any animal reaches this during the experiment, they will be humanely killed.

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

Non-mammalian animals are not suitable for the proposed work as they are not suitable hosts for

Toxoplasma gondii. The parasite will only grow inside cells at 37^{o}C. Mice are the least sentient animal that can be used as a model.

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

Mice will be monitored twice a day following infection with *T. gondii* and scored using a defined scoring system. There will be a pre-defined humane endpoint put in place, and any animals that reach the endpoint during the experiment will be humanely killed.

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

I will follow the current PREPARE and ARRIVE 2.0 guidelines on best practice and reporting on animal experimentation to ensure the studies are conducted in the most refined manner.

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

Being the secretary of the establishment's 3Rs Committee, I will be in regular contact with relevant people who can keep me informed of any advancements in 3Rs, including the Named Veterinary Surgeons, the Named Training and Competency Officers, and the Named Information Officers. I also receive email updates from NC3Rs (National Centre for the Replacement, Refinement and Reduction of Animals in Research) and FRAME (Fund for the Replacement of Animals in Medical Experiments) about matters relating to animal experimentation. I will also liaise regularly with the Named Animal Care and Welfare Officer to keep abreast of any new enrichment practices to ensure the mice have the highest quality welfare whilst on the study.

35. Neural mechanisms in health and disease, and therapeutic applications.

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, Cellular and molecular properties, Neuronal functions, Plasticity, Therapeutics

Animal types	Life stages
Rats	Juvenile, 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 primary aim of this project is to understand novel mechanisms involved in the function, assembly, and plasticity of neuronal circuits that control learning and memory, motor behaviour, and metabolism. These studies will help us understand how the brain processes information, generates behaviour, and performs various functions. Knowledge in this field will accelerate the development of new therapeutic strategies to treat brain diseases such as neurodevelopmental and neurodegenerative 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?

A better understanding of how the brain processes information and generates behaviour can help us identify the molecular mechanisms involved in brain dysfunctions, such as neurodevelopmental, neurodegenerative, and metabolic diseases. This program of work is crucial for developing new therapies, as no cures are currently available, and the existing treatments do not halt the disease's progression.

Huntington's disease, for example, is a rare genetic disease affecting 10:100,000 people in the

Western world. It typically affects adults who develop motor, cognitive, and psychiatric abnormalities for which there is currently no effective treatment. The burden on carers and health providers rises rapidly as the disease progresses, and it is estimated that care for Huntington's sufferers is 195 million per year in the UK alone.

Another example is late-onset Alzheimer's disease, which is the most common type of neurodegenerative dementia, accounting for up to 80% of dementia cases. It is estimated that 24 million people worldwide are affected by this form of dementia. Current treatments do not halt but only slow or delay the disease's progression. The overall cost to the UK health system is estimated at 26 billion per year.

Finally, metabolic diseases such as obesity and diabetes are typically linked to lifestyle choices. However, little is known about the role that particular neurons in the brain play in controlling food intake and energy balance. The health service is expected to spend up to 14 billion per year treating these diseases with ineffective treatments.

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

This work is expected to provide new fundamental knowledge about the molecular mechanisms that control the behaviour of nerve cells in functions such as learning and memory, locomotor activity, and cell metabolism in health and brain diseases.

We expect to refine factors identified with our previous research and discover new pathways that will help design novel strategies to prevent the disease's onset and/or more effective treatments.

The information generated from this project will be published in scientific journals. Any new genetically altered model will be shared with the community as appropriate. In addition, the discovery of novel potential drugs may result in patents.

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

In the short to medium term, our findings will benefit the scientific community that is interested in the fundamental molecular mechanisms underlying neurodegenerative, neurodevelopmental, and metabolic diseases.

In the long term, we expect pharmaceutical companies and, ultimately, human patients to benefit as there are no effective treatments for preventing or delaying these diseases or even improving symptoms.

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

Our results will be available to other scientists through publication in peer-reviewed journals and presentations at scientific conferences, meetings, and collaborations. This will allow us to share our novel findings nationally and internationally.

We will share any valuable new mouse models developed in this project for studies or tissues with collaborators.

Species and numbers of animals expected to be used

- Mice: 10000
- 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.

Mice and rats are good candidates for neurological studies as their brains can mimic the human brain.

The laboratory mouse, for example, shares a significant number (around 86%) of genes with humans.

The ability to manipulate the mouse and rat genomes means we can generate disease models that replicate human diseases, allowing effective translational analysis of the brain and metabolism.

To carry out longitudinal analyses of brain function and behaviour at several life stages of animals, neonates, juveniles, adults, and aged subjects are required.

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

Genetically altered animals will be bred using conventional methods. The mutations animals will carry are not expected to cause any harm that is likely to affect the normal behaviour and well-being of the animal. Some animals may exhibit a slightly wobbly gait, but it does not affect the animal's ability to eat, drink, and mate. Some mutations will be silent and only be activated by administering certain substances, typically Tamoxifen but also diphtheria toxin.

Some animals will be aged past 15 months up to a maximum of 28 months, as some of our diseases of interest are more prevalent in aged humans.

Some animals will undergo a series of behavioural tests to determine the effect of the genetic or neurological changes the disease causes. Most of the tests we utilise are non-aversive and do not cause any harm. For example, we use the rotarod test, where animals are required to stay on a slowly rotating rod for as long as they can. This time is noted as the disease progresses to see its effect on coordination. However, some of the tests are aversive. These tests include using a mild electrical shock to the foot linked with a cue. The level of the shock is typically brief and repeated up to 2 occasions in their lifetime. They may also be exposed to a water escape trial where they are expected to seek out a submerged platform. This is done in a shallow maze with warm water.

Some animals will have restricted access to food for motivation in behavioural tests.

To study the effects of the metabolism on neurological diseases, some animals will be fed an altered diet, which is higher in fat than their regular diet; treatment is only for a short

period, typically 3-4 weeks. Some of these mice will then undergo a glucose or insulin tolerance test to see how the altered diet affects these chemical levels in the body.

Some animals will have a small device implanted. This allows continuous delivery of drugs rather than undergoing daily injections. This involves minor surgery under general anaesthesia. Animals are given pain relief just like patients after an operation.

Some animals will be given substances that affect the normal behaviour of the neurons in the central nervous system, particularly the brain. The substances themselves are not expected to cause any harm, but some animals will need to undergo a surgical procedure to allow the substances to be injected directly into the brain. They may also be fitted with a small substance delivery device to infuse the substance rather than give it in a bolus.

Some animals will have a small blood sample taken so that we can monitor the levels of chemicals in their bloodstream.

At the end of the experiment, all animals will be humanely killed, and tissues will be harvested postmortem for further analysis in the laboratory.

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

Old animals can become unwell, just like old people can. Age-associated conditions in old animals include weight loss, reduced organ function, skin abnormalities, abscesses/tumours, eye disease, dental disease, joint disease, and mortality. Many of these conditions can be treated as advised by a vet.

Animals that have had surgery will experience some pain and discomfort and may temporarily lose a modest amount of weight (e.g. up to 15% of their starting weight) for a few days but are expected to recover quickly and will be given painkillers 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)?

Mice: 70% subthreshold, 20% mild, 10% moderate.

Rats: 60% mild, 40% 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?

Our study requires the use of a fully functioning mammalian nervous system to investigate the impact of the brain on disease progression. Mice are the lowest sentient mammalian model suitable for our purpose. Rats are also excellent for this purpose as, in some instances, they reflect the clinical signs of the diseases closer to that of humans.

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

Cell cultures or organoids, computer modelling.

Why were they not suitable?

Whilst we can use cell cultures and organoids can complement our work by allowing us to study effects on single cells, they are not able to replicate a fully functional mammalian nervous system. Computer models may give us some useful information about what might be expected to happen, but they cannot predict neuronal responses that may occur unexpectedly or processes that require ageing, such as neurodegeneration.

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 estimate is based on our previous experiments and our annual returns.

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

Generating precise and specific genetically altered mice results in more defined, less variable, and hence more relevant data. This correspondingly reduces the number of animals necessary to obtain significant results and improves the quality of life of the animals.

We follow the ARRIVE guidelines and NC3Rs' Experimental Design Assistant to plan experiments and to ensure blinding and randomisation in our experimental design, where appropriate.

If pilot studies are necessary to test a method, hypothesis, or dose range, they will be carried out using small groups of animals. Based on our previous experience, as few as 2-5 animals per pilot study are sufficient to reach a robust decision point.

Where necessary, control groups (including, in some cases, sham surgical controls) will be used to help us interpret the effects of experimental interventions. The number of animals undergoing surgical procedures in control groups will be kept to a minimum through (1) the use of ‘within animal controls’ (e.g. injection of toxin/agent in one brain hemisphere and injection of vehicle in the other hemisphere); (2) timely comparison of small groups of sham surgical controls with non-surgical controls to reach a robust decision point (e.g., if a difference is not detected, then it would not be necessary to use further sham surgical controls in that series of experiments); and (3) considering the available published evidence.

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 according to 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 stock and to assist in maintaining a suitable colony size to ensure only those animals needed for experiments are produced. Cryopreservation of colonies that are not required in the short term will be considered.

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 genetically altered animals to delete/activate genes of interest in specific cell types and/or at particular stages in the animals' lives. By targeting only specific cells, we avoid unwanted side effects.

We allow some animals to age to sufficiently model human diseases and assess the full extent of neuronal degeneration.

We use behavioural tests to investigate substances' effects on neural mechanisms. All these tasks are well established in the literature. In general, the procedures involved do not give rise to adverse effects. We do perform a fear-conditioning test that involves a mild foot shock. Sensory perception of the shock used in fear-conditioning has been determined in the past through shock threshold assessment, and the commonly used one gives rise to an annoying stimulus that is still enough to generate an associative learning process and covers the neural circuits of interest. This is typically on no more than two occasions during the animal's life.

Water or food restriction is used to motivate animals to perform tasks and causes minimal distress. Animals typically tolerate water or food restriction with no adverse effects. Body weight provides a robust health measure before any more severe signs of dehydration are observed, such as hunched posture or piloerection. This allows us to provide supplementary water as necessary to prevent adverse effects.

Animals are sometimes fed different diets, including high-fat diets, to study obesity. This helps researchers better understand the nature of abnormally increased appetite and/or food consumption. However, this only occurs for a short period before the animals are returned to their regular diet or euthanised.

We restrict food prior to glucose and or insulin tolerance tests, but this causes no adverse effects.

The administration of substances should not cause pain, suffering, distress, or lasting harm. Drugs/vehicles will be administered at established concentrations/volumes and via

routes most appropriate to animals, as shown by other investigators or our experience. They will be administered through the least invasive route.

In some experiments, we will administer substances using a device that allows for slow/steady dosing. This includes minor surgery, after which animals are closely monitored. This method is highly refined for repeated dosing.

In some experiments, we will administer substances using appropriate surgical methods (which include short-term surgery and an aseptic environment) and post-operative care (such as analgesia and easy access to food and water) to help minimise the occurrence of adverse effects.

The induction of central nervous system cell dysfunction by chemical means will likely change the animals' behaviour. We will regularly assess animals' behaviours, body conditions, and body weights after induction procedures. From the literature, it is uncommon for these behavioural changes to significantly affect the animals' ability to eat, drink, and groom.

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

While other organisms such as yeasts, worms and flies are excellent models for studying the cell cycle and many developmental processes, mice and rats are far better tools as they replicate a fully functional mammalian nervous system and are genetically manipulable to represent the human condition.

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

Animals will be closely monitored 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. For animals that have undergone surgery, we may provide additional measures such as moist food to ensure weight maintenance and medication to relieve pain or clean and heal wounds.

Handling 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 minimum distress to the animals.

Animals will be monitored during drug administration and 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?

- NC3Rs online guidelines, practical information, and other online resources.
- Laboratory Animal Science Association (LASA) provides advice on the care and welfare, education and training, ethics and policy, and regulation of animal research.
- Asepsis guidelines (LASA).
- ARRIVE and PREPARE guidelines.

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

We have registered on the NC3Rs website. We will regularly review their resources for best practices and integrate recommended refinements into our procedures as we identify them.

We will also attend internal 3Rs meetings and external conferences/seminars on innovative 3Rs techniques.

36. Understanding regulation of cartilaginous tissue formation, joint disease and regeneration

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

Key words

Cartilaginous tissue, Development, Cell phenotype, Joint disease, Therapy

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 understand the role different genes play in regulating the formation of cartilage within joints, as well as in maintaining joint health vs disease during ageing. The information gained will be used to help develop novel approaches that can be used to prevent degeneration or promote tissue repair.

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?

Joints, such as those in the knee and the intervertebral discs in the spine, play vital roles in allowing pain-free mobility. Conditions affecting these tissues, such as osteoarthritis in the knee/hip and chronic back pain caused by breakdown of tissue (degeneration) in the intervertebral discs, have a huge impact on societies around the world. For example, research by the World Health Organisation has shown that chronic low back pain is the leading cause of years-lived-with-disability globally and the cost of back pain in the UK is estimated to be between £14 and £28 billion/annum (1-2% of gross domestic product).

Likewise, osteoarthritis currently affects around 528 million people worldwide, an increase of 113% since 1990.

The mechanisms that underpin the development and maintenance of healthy cartilage versus disease are not well understood. As a result current treatments only treat the symptoms rather than the underlying disease and hence offer poor long-term efficacy. Research is thus required to better understand how healthy tissue is formed and what changes to cause joint disease, with a long-term aim of developing new treatments.

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

Improved academic understanding of the mechanisms which underpin healthy development and maintenance of cartilaginous tissues such as those in the knee/hip joint and intervertebral disc in the spine

Improved academic understanding of how disruption or loss of pathways that maintain cartilage tissue health might lead to joint disease

Development of novel approaches that can be used to prevent joint disease or promote cartilage regeneration

The knowledge will be shared through academic publications and presentations at national and international conferences.

Where appropriate knowledge will be shared with the public or patients through engagement activities.

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

The research will directly benefit the research group by enabling us to advance our understanding of genes and pathways identified in previous studies on cartilage tissue health. Other researchers both within the establishment and at other institutions will benefit through new opportunities for collaboration.

Knowledge will be shared academically at conferences during the project. These conferences are annual, so it is envisaged that data will be shared from year 2 onwards.

Findings will be submitted for publication as soon as practically possible. Ideally this will be from year 2 onwards, but may not be until the project has been completed.

Wherever possible, negative/null results (e.g. where genes are found not to play an important role in tissue formation, health or degeneration) will be published as this knowledge is important for other researchers to know to prevent similar work being repeated in the future.

In the long-term the findings from the study may be used to develop novel therapies which will directly benefit patients suffering from common and debilitating joint conditions such as osteoarthritis and back pain. Findings, such as the identification of key genes that prevent or delay disease progression, may take many years to reach patients, but we work closely with clinicians to ensure help identify potential opportunities for clinical translation of the research.

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

Key findings will be presented at conferences and submitted for publication in open access journals. This will include negative/null findings wherever relevant.

Any large datasets generated during the study will be made available to others by submission to publicly-accessible repositories.

The models generated in the project will open up opportunities for collaboration with other experts in the field.

We will engage with relevant patient groups and clinicians to ensure the clinical relevance of the research aligns to their priorities and needs.

We will also engage with the public through a combination of public engagement activities organised with the establishment, and through the dissemination of outputs through the media in conjunction with our Media Relations team.

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.

Mice allow for genetic manipulation, enabling the study of specific genes and pathways within a specific tissue. This approach is not possible in other species. By targeting specific genes and pathways within certain cells in mice, we can study their role in promoting formation and maintenance of healthy cartilaginous tissue and whether their loss results in joint disease. The study will also enable us to identify cells and pathways that can be targeted for prevention of degeneration or regeneration of cartilage tissue.

To achieve this work we will study mice across life course from birth to old age.

Target genes will be knocked out in specific cartilaginous tissues at defined ages to explore the function of our protein of interest in controlling either healthy tissue formation of maintenance of healthy tissue versus initiation of changes that lead to joint disease.

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

The generation of genetically modified mice will involve natural mating of animals. The mice will be kept in standard conditions for up to 15 months.

To induce tissue-specific gene knockout, mice will be treated with tamoxifen. This will be administered either orally or by injection.

To establish whether the genetic modification has been successful, mice will be tested using one of the following approaches: ear biopsies, blood sampling, hair sampling, or mouth swabbing. This will be done once, unless there are technical problems which mean it has to be repeated.

Mice will then be maintained over a period sufficient to assess the success of gene targeting and agerelated changes in cartilage tissue structure/composition, such as degenerate changes.

As well as examination of joint tissues after humane killing, tissues may also be examined using: noninvasive imaging methods, such as x-ray and MRI, and range of motion joint

analysis, no more than once per month; and by observational gait analysis (a maximum of once per week).

Control mice, e.g. without gene knockout, will be included for comparison in all elements of the project. Wherever possible, tissue from control mice will be used across different experiments.

Where required, animals with altered immune status will be housed in a barrier environment.

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

No adverse effects are expected as a result of the breeding of genetically modified mice.

Mice treated with tamoxifen can show a modest weight loss of <15%. This has been commonly observed and is transient and does not extend beyond the period of active dosing.

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 mice will be genetically modified and the breeding programme is a mild procedure.

50% of mice will experience a maximum of moderate effects, through tamoxifen treatment or development of joint disease. The remaining 50% of mice will experience a maximum of mild effects.

No animals will experience severe 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?

Cartilage tissues are complex, containing multiple regions that each have different structures that evolve over time. Such complexity cannot be replicated using in vitro models and human tissues are difficult to obtain at defined stages of development, maturation and healthy ageing.

A mouse model is essential for us to achieve our aims. Mouse joint structures and cartilage tissue composition are similar to humans and have been widely used as models for joint development, maturation and age-related joint diseases. They also grow, age and

develop joint diseases relatively quickly, allowing investigations over relatively short timeframes (up to 15 months).

Mice are also the ideal species for studying tissue-specific, targeted genetic modification. They will enable us to study the effect of editing specific genes and pathways on tissue health and disease progression over extended periods which is also not achievable in vitro, or using human tissues.

We will be able to establish healthy tissue formation, maintenance and development of joint disease at defined times using a range of non-invasive imaging techniques and end-point assays, all of which are routinely available. Such an approach would not be possible with human tissue.

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

We have already undertaken extensive investigation of human tissues from early fetal development, childhood (healthy tissue) and adulthood/ageing (diseased tissue). These investigations have enabled us to identify key genes/proteins which may be important for regulating healthy tissue formation versus joint disease. However, such investigation of human tissues does not allow function to be established.

To establish function of specific genes and to develop novel therapies we have considered:

In vitro investigations using human cells in which specific genes or pathways are edited.

Non-protected chordates such as zebrafish.

Computer modelling.

Why were they not suitable?

Human tissue does not allow gene function to be investigated and we have no control over the ages of samples studied, or their relative health or disease status. This makes investigations complicated, in particular where adult patients may have multiple other conditions in addition to joint disease.

In vitro models using human cells in which specific genes or pathways are edited can be used as a partial replacement used for screening before moving in vivo, but do not fully replicate the complex environment of cartilage tissues or the long-term changes that occur during development or joint disease.

Non-protected chordates such as zebrafish are useful for studies where genes or pathways are edited, but the size and structure of the cartilage tissues in such species do not accurately model human tissues, limiting the applicability of the knowledge gained.

Computer models have recently been developed to model some aspects of cartilage tissues. However, these models lack the complexity required to study age-related changes in tissue structure. They are also not suitable for the study of specific genes and pathways in the context of cartilage tissue development or degeneration.

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 that up to 2000 mice will be required for our project. These will be used in our breeding programme to generate mice where our specific genes of interest are knocked out, to study their role in cartilage tissue formation, maintenance of healthy tissue and development of joint disease, as well as to develop novel therapies.

This is based on: (1) the number of experiments we plan to use these mice for, and (2) the conservative assumption that each litter of mice we breed will include one mouse with the correct genotype.

For each gene of interest we estimate that up to 1000 of the mice that we generate in our breeding programme will be treated with the drug tamoxifen to stimulate the tissue-specific gene knockout. This is based on the different experimental time points we will use (in order to explore the effects of our protein of interest throughout tissue development, ageing and joint disease), and previous work by collaborators, which suggests that each experimental group will need to include 8 mice in order to generate informative data.

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 outcomes of work in publications and our collaborator's labs, and have taken the advice of a statistician to reduce the number of mice being used in each experimental group. We will use pilot studies to further adjust group sizes as the study progresses.

By using both male and female mice in our studies, and by utilising efficient breeding strategies, we will reduce the number of genetically modified mice that we need to breed. Furthermore, generating data in both male and female animals is important with regard to the translation of our findings towards human clinical studies, where joint disease is common in both women and men.

We will also ensure that tissues from control mice, e.g. without gene knockout, are used across different experiments.

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 continually review the outcomes of experiments and use our data to optimise animal numbers as the project progresses. For example, we will conduct pilot studies to optimise the protocol for tamoxifen induction of tissue-specific gene knockout and to determine the group sizes required in 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.

This project will selectively knock out genes of interest in cartilage tissues of mice at different stages of development and ageing. We will use these models to explore the effects of the gene deletion on healthy tissue formation and maintenance during ageing versus spontaneous development of joint disease. The information will be used to develop novel therapies to treat joint disease.

Given the slow-progressing nature of joint diseases, some animals will be allowed to grow old to study the changes that occur in cartilaginous tissue structure and composition. Any effects are likely to be mild to moderate and we will minimise the time animals experience joint disease.

To induce or knock out gene expression in animals or to deplete specific cells, some animals will be given tamoxifen by mouth, injection, or through food. Any effects will be mild and transient and we will optimise the dose and duration of treatment.

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

In order to explore the onset and development of human joint disease we need to use a live animal model with a mature musculoskeletal system that is similar to an adult human. The adult mouse fulfils this requirement. Non-mammalian animals are limited in their use because their musculoskeletal structures are too different from the human and their lifespan is too short to allow age-related changes to be studied.

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

The major welfare cost to the mice in this study is the development of joint disease symptoms. Since the purpose of this project is to study the effect of genes in regulating formation and maintenance of healthy cartilaginous tissue versus joint disease, this welfare cost cannot be changed. However, we will not investigate genes which are known to have severe impacts on human health and will limit the time animals experience disease whilst giving analgesics where appropriate.

We will carry out pilot studies to determine the least harmful way of administering tamoxifen, whilst still ensuring effective knockout (determined by genotyping). We will preferably use an oral method of administration. Tamoxifen treatment can cause some weight loss and signs of general malaise such as loss of appetite, hunched posture and piloerection, but these are typically transient.

As well as collecting data on joint damage and gene expression profiles at the end of each experiment, we will use non-invasive methods (such as imaging and observation of animals' mobility) during the course of each experiment so as to maximise the information we obtain, but without any additional welfare costs to the mice.

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 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 abide by the recommendations of the joint working group on refinement (Morton et al, Laboratory Animals. 2001;35:1-41) and use published documents recommended by NC3Rs to ensure that we are using the most refined approaches in all our experiments. We will also regularly review the scientific literature for work by other researchers in the field to identify opportunities for refinement.

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, we will meet the establishment's 3Rs manager, and attend regional 3Rs symposia.

37. Immune responses to infection and vaccines

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, Cancer, Infectious disease, MAIT cells, Immune memory

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 project aims to investigate how the immune system responds to pathogens (such as bacteria and viruses), cancers, and vaccines, and to use these insights to develop improved therapies and new vaccines.

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?

Infections and cancers are major human health threats. For infections, we understand that immune responses are crucial, and vaccines have proven to be one of the most effective medical interventions ever developed, playing a pivotal role in managing the COVID-19 pandemic. However, we still lack vaccines for many important infections such as hepatitis virus, HIV, and herpes related viruses such as Cytomegalovirus and Epstein Barr Virus. In recent years, the ability of the immune system to protect against cancers has also been revealed, but we have not fully used this potential for many types of cancer. To develop more effective vaccines and immune-based therapies for these diseases, we need a deeper understanding of how immune responses generate protective immunity.

While our primary focus with vaccines and immune therapies is on viral infections and cancer, emerging challenges from bacterial infections are also a key concern. Each year many people around the world die from bacterial infections and increasing rates of antimicrobial resistance (AMR) indicate an emerging crisis where infections are becoming harder to treat and may become untreatable. In low and middle-income countries (LMIC) with high rates of AMR, access to antibiotics to treat these infections is difficult. The World Health Organization has recommended research on developing vaccines to prevent or reduce the impact of these infections as an important priority.

Most of the work trying to understand mechanisms of immune protection focus on classical parts of the immune system, such as B and T cells (which are key in enhancing immunity) and the structures within which they reside. In recent years, we have learnt that the brain also plays an important role in how our immune system works. The brain senses, regulates, and remembers immune signals that suggest infection somewhere in the body. We do not yet know how these responses might be changed by neurodegenerative diseases, such as dementia. We know that people with dementia get acutely confused when they experience infections that other people can fight off easily. By studying the immune responses (antibodies and T cell) following vaccination of mouse models of neurodegeneration we aim to recognise such a deficiency and identify treatments that could be used in people.

The same processes that generate effective immune responses against viruses can also impact on cancers. We are interested in the role of a particular subset of T cells called MAIT cells, which appear to play a variety of roles in boosting immunity against infections and cancers and may play a role in the response to immune therapies. Although we can study such cells in humans and try and understand their behaviour, by depleting them or adding them back in mouse studies we can understand whether they are playing an important role or not and how this may impact on therapy in patients. We hope to be able to design better therapies in the future.

Overall, the aim of the project is to examine in detail how the immune system combats viruses, bacteria, and cancer, to understand how vaccines can enhance these defences, and to apply this knowledge to develop more effective interventions across various settings.

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

New information that will help to understand the nature of the immune responses to pathogens (e.g. bacteria and viruses) and vaccines, the role of specific new types of immune cells, and how we may harness these responses to tackle cancer. The information on how best to use the vaccines can be directly used to design better strategies (types of vaccine or combinations of vaccine) in different infections and cancers. We aim to publish the results in peer-reviewed journals and at conferences.

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

The science community focused on infections and cancers will be interested in the new information we will generate regarding how the immune system responds to vaccines and pathogens. For example, the role of MAIT cells (which may boost immune responses) and related cells, has not been well explored in this context but has a lot of potential impacts. We also hope to answer questions relevant to the use of vaccines in cancer therapy to help design trials in patients for cancers such as melanoma. These trials and vaccines are being designed over the next 2-3 years and our results can help contribute to making the best vaccines - or the best use of vaccines - from the different choices available. Longer term, understanding of the basic mechanisms of how the immune system responds to

vaccines will help us design vaccines against new threats, and/or with induction of longer memory and therefore longer-lived protection.

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

We have collaborated widely to improve the outputs of the work and have colleagues in labs across the world whom we will share our data. This includes academic colleagues in universities as well as collaborators in biotech companies aiming to develop vaccine strategies. We will also share our data at scientific conferences in immunology, infectious disease, and cancer immunotherapy, and publish our findings in peer-reviewed journals. The data published will include the relevant negative data as well as positive results which can be followed up by the field. Wherever possible, we will share the tissues and foster collaborations to maximise the output obtained from the project.

Our lab does a lot of public engagement - including with schools - so we aim to spread understanding of the immune system and its role in infections and cancer widely.

Species and numbers of animals expected to be used

- Mice: 12700

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 will be used in this project because these are considered to represent the fully developed immune system of humans. There are well defined models and reagents readily available. For vaccine studies there is a good pathway for taking the results from mouse studies into humans - as was done recently for the Covid-19 vaccines.

We will use both wild-type mice and genetically modified animals engineered with specific defects in immune signalling, alterations in threat recognition by the immune system, or modifications to model aspects of neurodegenerative disease.

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

Most animals will receive two injections (e. g. immunisation with vaccine) followed by up to four blood tests taken over a period of 8 weeks to test the effect of the immunisation.

Some animals will then undergo a pathogenic challenge after an initial injection. This consists of an infection or disease caused by harmful microorganisms like bacteria or viruses.

To increase the number of MAIT cells (cells that boost immunity), some animals will be infected intranasally with Salmonella or injected with other small molecules.

Some animals will have a tumor implanted subcutaneously, either before or after the initial vaccination, and will be monitored non-invasively. Additionally, some animals will receive an anti-cancer compound (e.g., antibodies) or a substance designed to enhance the immune response to vaccination before being challenged with a tumour.

To test how the immune response is working to protect against an infection or cancer, some animals may receive an additional injection with substances that will allow the analysis of specific immune pathways. For example, with specific immune cells/cytokines (signalling proteins) or substances that deplete a subset of the immune system (e.g. antibodies).

To allow targeted gene deletion, some animals will receive a substance that enables the study of specific genes' roles in immune function, development, and disease processes.

Some mice will undergo low-dose irradiation to eliminate their bone marrow, after which they will receive bone marrow from a different mouse strain to replenish the depleted marrow. This procedure is similar to bone marrow transplantation in humans, used to treat blood and immune system disorders that impact the bone marrow and allows to track specific immune system components. To prevent opportunistic infections, antibiotics may be administered, usually for up to 28 days around the time of irradiation.

All animals will then be killed humanely before one year of age.

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

After immunisation, mice may experience a short period, typically no more than 24 hours, of signs of general malaise such as hunched posture, piloerection, and lethargy due to over stimulation of the immune system. This is like the effects experienced by humans after vaccination.

Animals will experience mild and transient discomfort from blood sampling.

Animals that undergo the induction of a subcutaneous tumour may experience weight loss of up to 15% compared to their baseline weight, at which point they will be humanely killed. As soon as their body weight drops to 10% from baseline, we would support these animals with moist palatable food to ameliorate the weight loss. Typically, we would expect to see improvement within 24 hours of this intervention. If recovery of body weight is not observed, the animal will be humanely killed.

Subcutaneous tumours might invade the surrounding skin or muscle, potentially affecting normal movement. Some tumours may ulcerate, typically presenting as a dry scab, but occasionally a wet ulcer could develop. In such cases, treatment will be administered based on veterinary advice. If there is no sign of improvement within 24 hours, animals will be humanely killed.

Animals that undergo a dose of sublethal irradiation will experience a temporary suppression of their immune system, making them more susceptible to infections until the bone marrow transplant starts to rebuild their immune defences. Adverse effects may include a transient weight loss of up to 15% between days 7 and 10 after irradiation, which typically resolves by day 14. If their body weight drops to 10% from baseline, we would support these animals with moist palatable food to ameliorate the weight loss.

If an animal loses 15% of its bodyweight it will be humanely killed, except following pathogen challenge where the humane endpoint is 20% bodyweight loss.

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)?

Subthreshold 36%, mild 44%, 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?

Animal models are required to study the mechanisms of immune responses during vaccination and infection because in vitro models do not fully recapitulate the immune system required to evaluate vaccines. Mice are the most relevant mammalian animal model because of their genetics and immune system, which is similar to that of humans.

Our lab mainly studies humans with infection and cancer following administration of vaccines. In all these cases we focus on measuring the immune response as carefully as possible to understand its function. However, we cannot tell what the most important part of the protection is unless we first perform experiments in mouse models which help us create better therapies. Further, we cannot create new or improved vaccines and therapies for humans based on this understanding unless they are tested carefully first in mice to understand how they work.

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

We are developing models to study some of the simplest elements of virus persistence in cell culture. For example, we have set up infectious model systems for Hepatitis B and C virus where intracellular responses can be assessed and where interactions between the virus and immune system can be modelled. If we have further indications from these cell culture models which could lead to replacement of the planned in vivo work, we will take advantage of this. We are also working on "organoid" models (that is, tiny cultures of tissue which can represent some aspects of the organ which they came from). One of the best exploited is that of human tonsil where some aspects of immune homing (where the body detects an infection or injury and sends out signals to attract immune cells to a specific location) can be measured in vitro. We are using these systems to answer some of the questions we are interested in.

The lab works with human studies including analysis of Phase 1 clinical trials. The experiments planned are only those where we cannot achieve the same objectives by analysis of human subjects. These trials have focused on the development of prevention and therapy of Hepatitis C Virus and for protection against Respiratory Syncytial Virus (RSV), both of which both of which contributed to the development of the Oxford-AZ COVID-19 vaccine. We have ongoing studies of human immune responses to SARS-Cov-2, Hepatitis B and C virus, HIV and Cytomegalovirus in the lab, studies of cancer responses, as well as analysis of human innate T cell responses such as MAIT cells which can control bacterial and viral infections in tissues. These data have been used to plan better experiments in the mouse model, which can then be used to address questions most relevant to the clinical setting.

Why were they not suitable?

Such ex vivo experiments cannot address the immunogenicity (how well a substance, like a vaccine or a drug, can trigger an immune response in the body) of a range of vaccines - we can only test very few vaccines in humans at a time. To define which of the potential approaches might be best (and safest) we still require an intact and flexible in vivo system. It also needs such a system to assess the efficacy of induced responses against challenges such as pathogens or tumours. Finally, we cannot fully address the mechanism of action of specific components of the system (e.g. by deleting specific cell types or using genetically modified animals) unless we use an in vivo model where they are lacking or modified. Therefore, while the key studies in the lab will remain focused on human studies, we still very much rely on the mouse models to make progress in this area of immune protection.

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 estimates are based on the usage data from previous projects.

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

In many cases, the numbers of animals required will be reduced by repeated, longitudinal measurement of responses through serial blood analysis. The immune response to an immunisation may be measured weekly in a set of five mice over a period of several weeks rather than killing five mice every week. This approach also provides valuable information on the development, or not, of protective immune responses prior to challenge with a pathogen or tumour. We will be using the NC3Rs' Experimental design assistant which we have used during our previous licence.

Using tools like antibodies and other large molecules (tetramers) that can target and remove specific types of immune signals or cells will allow us to conduct experiments in wild type mice, reducing the number of genetically modified animals we would have to breed for these experiments.

The methods in the lab use "transcriptomic" techniques, which use RNA sequencing approaches.

These are very powerful and allow much more data to be generated from smaller numbers of animals (by studying thousands of cells per animal in enormous depth). These new approaches allow us to do fewer but more powerful experiments, using less animals.

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

Animal numbers will be optimised by purchasing wild type mice for specific experiments rather than breeding them. Genetically modified strains bred in-house will be closely managed and both sexes will be used in experiments.

We routinely use many tissues from a single animal at the end of the experiment instead of using individual animals to obtain each tissue of interest. This overall reduces the number of animals required.

Occasionally we will share tissues with other members of the group to perform in vitro assays, microscopy and genetic analysis.

We do pilot studies in a small number of animals to validate pathogenic/tumour challenge doses, vaccine regimens and doses before conducting larger 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.

We will use adult wild type mice. In some cases, genetically modified mice will be required. These wellcharacterised models will aid in deepening our understanding of how different vaccine strategies enhance immunity against pathogens and cancer or how immune responses may be diminished by neurodegeneration.

Animals will undergo administration of substances. Whenever possible, the least invasive route of administration will be chosen. If intravenous injection is needed, both lateral tail veins will be used to minimise the risk of vein damage.

Blood sampling is expected to cause only mild and transient pain.

The induction of tumours will follow best practice guidelines and animals will be monitored to minimise suffering, distress, or lasting harm. In some cases, tumour induction will be performed under anaesthesia to allow for better positioning of the tumour.

We will use the minimal irradiating dose needed to achieve bone marrow depletion. Using two lower doses with a resting period in between also reduces radiation sickness for the animals.

Animals undergoing an infectious challenge will follow best practice guidelines and will be monitored to minimise suffering, distress, or lasting harm.

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

Mice currently offer the only small animal model of human disease with sufficient physiological similarity to humans, particularly in terms of a comparable immune system. They also benefit from a wealth of existing resources (e.g. mouse specific reagents) and the ability to mature rapidly. A similar mammalian immune system is essential for human immunity studies, especially for vaccines, to accurately model and define the complete immune response post-vaccination. The structural replication of the human immune system, especially the lymphatic system and its lymphoid organs in mice, underscores the importance of using this model. The mouse immune system is the model most suitable for human immunity studies, particularly for vaccines. Only mammalian immune systems

possess all the necessary components, and the well-defined structural composition (lymph nodes and organs) needed to accurately define the totality of the immune response.

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

The mouse is a very well-defined model for immunologic experiments and the models proposed are highly reproducible and therefore can be used more efficiently and with the maximum refinement. For example, we have developed the use of the non-replicating adenovirus model (which are those that don't trigger the same cellular responses because they don't replicate the viral genome and lack the ability to cause cell damage), which in turn has less impact on the animal.

We have minimised the amount of blood drawn per time-point by optimising lab immunology protocols and minimised the numbers of bleeds required per experiment. Using a variety of precisely engineered genetic models with specific immune system defects to study how antigens are presented and how T cells are activated and directed makes the mouse model particularly suitable.

The use of a range of precisely engineered genetic models with specific immune defects to study the how antigens and T cells are activated reduces the chance of encountering a harmful phenotype.

Increased habituation of mice in long term experiments that involve repeated blood sampling and administration of substances will be implemented with guidance from the NACWO and NC3Rs resources.

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

The NC3Rs website provides guidance to best practice through e-learning, blogs and videos. Information to aid design and planning of experiments is also available, particularly the experimental design assistant.

Guidelines for the welfare and use of animals in cancer research by British Journal of Cancer.

Researchers using protocol 3, Immune induction-tumour model, will join the NC3Rs Oncology Network to maximise the scientific and 3Rs impacts of oncology models.

Relevant LASA publications https://www.lasa.co.uk/current_publications/

OBSERVE: guidelines for the refinement of rodent cancer models: Nature Protocols July 2024

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

We have signed up to the NC3Rs newsletter and attend regular internal 3Rs meetings to keep ourselves updated on best practices. Additionally, we will enhance our skills and knowledge by attending workshops, seminars, and training sessions focused on improvements in 3R methodologies.

Our institution hosts an annual 3Rs Day, where internal and external speakers from diverse fields present their work and share advancements in the 3Rs.

38. Modulation of neural circuits for 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, head direction cells, navigation, tauopathy, nerve cells

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 is to define the kinds of brain cells that provide us with the ability to navigate environments, which involves remembering routes and locations. This is collectively referred to as spatial memory. Some of these brain cells, especially those that provide us with a sense of direction, become gradually impaired during ageing and are severely impaired in neurodegenerative diseases such as Alzheimer’s disease.

To investigate spatial memory in ageing and disease, we will control (modulate) the activity of subpopulations of nerve cells while recording the activity from connected brain regions. We will focus on brain regions that play a significant role in spatial memory, such as the anterior thalamus, retrosplenial cortex, hippocampus, and entorhinal cortex.

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?

Creating, storing and recalling spatial memories is fundamental for survival as it helps us anticipate novel experiences, find routes to food sources and shelter, and make

predictions on how to act (e.g. avoiding predators). By studying these processes in rodents, which have very similar brain structures and nerve cell connections to humans, we can define how spatial memories are distributed in the brain and gain insight into what happens when this process starts to break down in progressive neurological conditions such as Alzheimer's disease.

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

We are working on brain regions that are known to be affected in neurodegenerative diseases such as Alzheimer's disease, which include the anterior thalamus, retrosplenial cortex, hippocampus, and entorhinal cortex. These areas are vital for memory, and are important for finding our way around and for remembering locations and events. We will define the activity, kinds of nerve cells, and connectivity of these brain regions to increase our understanding of how memories are stored and recalled.

We will compare data obtained from mice (e.g. models of neurodegenerative disease) to data obtained from dementia patients and healthy controls. These will include data on the distributions of proteins that mark different populations of nerve cells and the presence of pathological (dysfunctional) proteins that indicate the progression or severity of the disease. Other data include new information on the activity patterns of the different kinds of nerve cells contributing to memory in connected brain regions. We will also obtain data on the behaviour of mice during tasks that test their memory.

Valuable scientific (neuroscientific and neurological) data will be collected, advancing our understanding of the mechanisms involved for creating, storing and recalling spatial memories, discovering and defining new nerve cell types, and also gaining much needed insight into how these processes break down in the stages leading to dementia.

Valuable data collected from animals will be posted on preprint servers and then published in openaccess peer-reviewed journals. The multidisciplinary approach we use to study the brains of both rodents and humans will benefit the neuroscience community as well as clinicians and patients through access to these data in published form but also via other outputs such as public talks and seminars, posters and social media.

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

The immediate benefits will be to the neuroscience and neurodegeneration fields. The outputs can be rapidly disseminated at conferences and in the form of preprints before peer review. Novel observations and new insights relating to the cell types critically involved in enabling mammals (including humans) to encode, store and recall spatial memories will be used by other laboratories and scientists to advance their own research.

If we discover changes in aspects of spatial memory when conducting behavioural tests in mouse models, we may be able to use these results to find early cognitive biomarkers for dementia. This will promote research on human navigation and memory. This will then help physicians identify patients that are at risk of developing dementia, leading to early intervention such as promoting lifestyle changes.

Our findings will benefit the public, e.g. through outreach events, explaining how and why we do research. If we discover how particular proteins implicated in neurodegenerative diseases directly affect nerve cell activity, this information will benefit patients, medical professionals and pharmaceutical companies. This may take a long time (3-5 years) due to the different avenues of investigation required to understand mechanisms underlying spatial memories.

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

- Research results will be disseminated by publishing them in reputable journals in open access format, so that they are available to all

- Results and knowledge will also be disseminated through public lectures and poster presentations in scientific meetings and in teaching

    - Open days, outreach events, science festivals

    - Collaborations with other groups will be used as a way to efficiently share knowledge and expertise

- International exchange programmes, whereby scholars and students from other countries visit our research group

- Social media and press releases, videos

Species and numbers of animals expected to be used

- Mice: 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.

We are mainly using adult and aged mice, and possibly some juvenile mice. We will also breed some genetically-altered mice, which requires pregnant females. Mice are an excellent species to work with because we know a lot about their physiology and anatomy, which is very similar to humans. They adapt very well to the experimenter (as with pets) and scientists have tools specifically designed to be able monitor brain activity that work very well with these animals. The vast majority of animal experiments in research institutions use mice, so the infrastructure (housing, husbandry, expertise of vets etc.) is optimised for their welfare. Furthermore, genetically-altered mice have been bred that mimic some aspects of neurological diseases, such as Alzheimer's disease, so this makes these animals ideal as models for human diseases.

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

Studying brain activity at the level of individual nerve cells, and in populations of nerve cells in different brain regions, requires surgery in order to implant miniature devices that can detect brain signals. These surgical procedures are similar to those done for human patients, in that mice are given an anaesthetic to put them to sleep and block any pain, then the skull is partly exposed, and extremely fine wires are lowered into the brain and secured in place with special bone cement. Sometimes we will inject into specific regions of the brain a non-harmful virus that will express specific proteins in groups of nerve cells. Surgeries typically last 1-2 hours and the animal recovers in its home cage within ~24 hours. Some more advanced surgeries will last longer (e.g. implanting more complicated recording devices).

For experiments, mice have their memories tested by letting them do some behavioural tasks such as running in mazes for rewards, or swimming for a few seconds to a hidden

platform in a particular location. For the animals that have undergone surgery, they will be connected to cables so that we can monitor brain activity on a computer while they do behavioural tasks, simply explore an environment, or sleep. In some experiments, we will shine light inside the brain using a fibre optic cable, and this light is able to activate the special proteins that are expressed by the virus that was injected during the surgery. This light activation causes the nerve cells to become activated and we can measure what happens to other nerve cells after light delivery. By implanting a mouse with a small plate attached to the skull, we can temporarily restrain them by the head while they are free to voluntarily move and rest on a running disc. Mice adapt very well to this and it enables us to gain direct access to the brain using specialised probes (such as glass electrodes), in order to accurately record activity of nerve cells from small regions of the brain that would be very difficult to target otherwise.

In some cases, mice undergo mildly aversive behavioural tasks to test different aspects of their memory. These tests include using a mild electrical shock to the foot linked with a cue, for no longer than 2 seconds. They may also be exposed to a maze or pool filled with shallow water where they need to seek out a platform that is slightly submerged. In some cases, animals will be exposed to mildly aversive stimuli such as sounds or smells.

Some animals will have restricted access to food or water for a few days or weeks for positive motivation in behavioural tasks. Some animals will receive a diet that is supplemented with or deficient in certain nutrients to test their influence on memory processes.

We will use genetically-altered animals that have been engineered to express human proteins that are implicated in neurodegenerative diseases such as Alzheimer's disease. The expression of these proteins in the brain affects memory, so that genetically-altered animals show deficits in memory that increases with age. We want to keep these animals for many months, including until they are 'aged' (up to 28 months), so that we can track how the brain circuits directly related to memory are gradually affected and understand the mechanisms involved in their degradation. For the vast majority of these genetically-altered animals, their daily life is unaffected and the memory impairments do not impact their welfare.

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

Surgical procedures cause pain, so we always give appropriate anaesthetics for pain relief before starting the surgery. It is also important that we provide additional analgesia at the end of the surgery that will last for at least a day after the operation, so that animals can recover properly. Most animals return to normal within 1-2 days. However, in rare cases where they take longer to recover, we would give appropriate drugs to reduce further adverse effects.

To positively motivate the animals to do certain behavioural tasks like learning how to find a reward in a maze (testing their memories), we may need to temporally reduce their diet or water intake. This means that they lose weight (typically up to 10% of starting body weight) while they are being trained and tested, which we will monitor by weighing them at least once per day.

Water-based tasks are commonly used by researchers to test spatial learning and memory abilities without any pre-training and reduce the influence of other variables. These tasks do not lead animals to exhaustion. Typical swim durations are very brief (~5 seconds in the water per trial), so it is very unlikely that water-based task testing could result in

hypothermia, and this has never been observed before. Swimming times and trial numbers are limited.

For aged animals (up to 28 months old) we expect some to develop age-related conditions, much like humans do. These conditions will be monitored closely and animals will be used for experiments at the earliest stage possible. Diseases such as Alzheimer's disease often appear in old age, so it is sometimes more appropriate to investigate aged mice as they will be closer to the human condition.

For a minority of genetically-altered animals that have been engineered to develop memory impairments, a particular line may show some minor impairments in motor coordination that increase with age (e.g. gradual loss of hindlimb movement). This is a consequence of the type of genetic alteration. It is very unlikely we will need to investigate these animals, but if we do, we will only use this type of animal if no other is available, and wherever possible the scientific information will be obtained before significant motor impairments develop.

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)?

Breeding protocol: 100% mild

Other protocols: 80% moderate, 20% mild or subthreshold

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 anatomy, neurochemistry and physiology of a particular region of the brain, or indeed individual neurons in a particular region of the brain, can only be established by the analysis of the brain itself and thus requires the use of animals. We need animals to be able to look at brain activity, how particular neurochemicals affect memory, and how this activity relates to neurodegenerative diseases.

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

In addition to studying nerve cells in human brain samples in related non-animal projects, we monitor the progress of the use of *in vitro* methods, such as organotypic co-cultures, to determine whether they can substitute for at least some of the *in vivo* work. We are also keen to explore *in silico* methods (advanced computer models) by initiating collaborations with computer scientists.

Why were they not suitable?

The use of *in vitro* methods and computer models cannot replace the use of animals because the questions we ask require behaviourally-relevant activity patterns involving large populations of brain cells with specific inter-connectivity which are expressed in the individual. These particular patterns and connectivity cannot be replicated in cultures either.

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 estimated the number of genetically altered animals generated through breeding and how many would be genetically altered (heterozygotes) and how many offspring would be non-transgenic. For animal experiments involving surgery (which includes the genetically altered animals), we based the numbers on previous licences involving similar procedures, the success rates for obtaining scientifically-relevant results, the difficulty of experiments, and the time investment in each animal relative to the number of researchers and their capacity.

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

Following expert statistician advice and taking assistance from the NC3Rs' Experimental Design Assistant, as well as designing experiments compliant with the Medical Research Council guidance with optimal sample sizes and high precision allowing within-subject and unbiased between-subject comparisons in order to achieve maximal statistical power.

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 monitor closely and implement efficient breeding, test new conditions using pilot studies and will be reusing tissue from the same animals for histological and electron microscopic investigations.

We will test antibodies as far as possible on tissue sections taken from the human brain.

Much of our work involves analyses in which we study molecules that are present in preserved tissues. We store these tissues for future studies.

Our multidisciplinary approach by combining physiology/tract-tracing/histology in individual animals enables far more data to be obtained from single animals. These combined experiments will be planned carefully for maximum scientific output with the minimum of cumulative effects.

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 use of wild type and genetically-altered mice will allow us to take advantage of the wealth of information already available, giving us the specific or selective access to certain cell types that we need to carry out the proposed functional investigations.

An example of a genetically-altered mouse that we may use is a model for neurodegenerative disease that expresses a human version of the tau gene (linked to Alzheimer's disease) so that we can study how the expression of the gene affects brain activity in mice, which will help us predict its effects on the human brain.

To study a particular pathway in the brain we may inject a virus that expresses human tau into a specific brain region to label a specific subpopulation of cells, then follow-up the effects of its expression over the lifetime of the animal. This is a refinement to the use of the more popular genetically-altered mice as there are fewer unexpected changes in their genes.

Diet/water restriction is commonly used as a reliable positive motivator for performing behavioural tasks. Most behavioural tasks we use have an appropriate level of difficulty and have been used for decades in rodents to study different aspects of memory, some of which are dependent on the very brain regions we study (such as the hippocampus).

Surgeries are conducted only when necessary. For example, behavioural tests can be conducted before surgeries, and mice are given time to adjust to having an implant.

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

It will be necessary to carry out the experiments on mammals rather than reptiles or fish because other groups of animals do not have a brain structure sufficiently similar to humans.

The cerebral cortex and related structures in the brain are relatively conserved in mammals. Mice are one of the species about which we know most and are thus considered the least sentient vertebrate group in which to carry out experiments. Data from this species are directly relevant to human brain function and dysfunction.

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

Good surgical practice and aseptic surgery will be carried out using appropriate drugs to minimise any adverse effects of the surgery. Animals will be frequently monitored during post-operative recovery until being able to engage in species-specific behaviour. For behavioural tests, we will aim to use the most refined test that minimises stress. For example, depending on the type of memory being tested, a water-filled Y-maze task is preferable to an open field swimming task because animals would spend less time in the water in the Y-maze.

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

Animals in Science Regulation Unit, Annual Report.

NC3Rs reports.

ARRIVE Guidelines on study design and reporting: https://arriveguidelines.org

LASA Guidelines on dosing routes and volumes:
https://pubmed.ncbi.nlm.nih.gov/7779463/

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

We subscribe to the NC3Rs newsletter, attend Institutional/Departmental Animal Welfare meetings, attend animal welfare events (e.g. NC3Rs Research Days), and maintain excellent communication with our Named Information Officer, animal technicians and veterinary surgeons.

39. Cerebrospinal fluid circulation and neurodegeneration

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Neuroscience, Neurodegeneration

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 understand how cerebrospinal fluid circulates in the brain clearing out waste products which drain into the meningeal lymphatic vasculature. We will also study how immune cells monitor those clearance routes interacting with protein aggregates in the context of 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?

Neurodegenerative diseases are a heavy burden for individuals and society as a whole. By 2016, the global number of people with dementia was estimated at ~44 millions, and this number is clearly on the rise. It is estimated that there are over 850,000 people living with dementia in the UK and that this costs the UK £26 billion a year. The project will address key aspects of dementia in a highly interdisciplinary way, aiming to clarify how the brain

disposes of protein aggregates which are thought to be responsible for diseases such as Alzheimer's and Parkinson's.

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

The main output will be in form of scientific publications. We will present this work in national and international meetings as well.

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

To animals:

The project aims to understand how cerebrospinal fluid (or interstitial fluid, i.e. the liquid in between cells in the brain) flows inside the brain and drains to lymphatic vasculature in the meninges, disposing of toxic protein aggregates. The clearance of protein aggregates is an issue that affects animals in general, not only humans. Mounting evidence suggests that there are a variety of species that suffer different forms of amyloid and/or tau pathologies, including domestic dogs, domestic cats, a variety of non-human primates, squirrels, black bears etc.

To humans:

Humans will benefit of scientific breakthroughs arising from this project that could find regulatory mechanisms to keep the brain free of the accumulation of toxic protein aggregates which are responsible for diseases such as Alzheimer's, Parkinsons etc.

To scientific knowledge:

We will provide novel experimental evidence in order to clarify the current controversies on how cerebrospinal fluid circulates in the brain and how it drains into the meningeal lymphatic vessels, clearing waste from the brain. This is a fundamental issue in the field of neurodegeneration, in particular in Alzheimer's disease research.

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

Our work is highly collaborative. We will collaborate and share results with top leaders in the field, including my collaborators in Europe and the UK. We will publish both successful and unsuccessful results and disseminate them in conferences.

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.

In order to study how the cerebrospinal fluid circulates in between cells and naturally clears out toxic agents involved in neurodegenerative diseases, we need a model of study that has the physiological mechanisms relevant to this process. Ultimately, this can only be achieved using living animals, at ages in which the barriers between compartments in the brain are already formed and fully functional. The model organisms to be used will be mice, including transgenic Alzheimer's disease mice and their corresponding mice controls which are not genetically altered, since they have a suitably advanced nervous system for investigating the mechanisms we want to address. Transgenic mice models recapitulate some of the main features in Alzheimer's disease, which will allow us to understand how the disease affects the mechanism under study in this project. We will use animals ranging from juvenile to1 year old adults. Most experiments can be done in young animals, but a small fraction of the experiments with transgenic and their wild type counterparts will require animals of ages between 9-12 months old to allow the development of the Alzheimer's phenotype.

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

The project requires the injection of tracers (small fluorescent tracers, nucleic acids or proteins), subsequently imaging the movement of those tracers to describe the circulation of cerebrospinal fluid. Imaging of the brain and cerebrospinal fluid will be done with a variety of microscopy techniques, which require the preparation of brain and cerebrospinal fluid samples as well as the direct observation of the brain in a living (anaesthetised) animal through a cranial window.

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

The injection of substances (small fluorescent tracers, nucleic acids or proteins) to the central nervous system will require a brain surgery under anaesthesia. Only 10% of the animals will be allowed to recover from anaesthesia, and will be given the necessary analgesic doses and will be closely monitored. As all the substances that we need to inject are biocompatible within the timeframe of the experiments (up to 4 weeks post injection) we do not expect any abnormal behaviour. The observation of the living brain will require a cranial window, and will also be done under anaesthesia. For the transgenic animals that will model Alzheimer's disease, we a lower body weight when compared to wild type 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 Protocol 2 (injection of substances to the brain under anaesthesia), 10% of animals will be allowed to recover, thus this protocol will be classified as moderate severity. The other 90% will be nonrecovery.

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 study the circulation of cerebrospinal fluid in the brain we cannot use cultured cells, as the native architecture of the brain is needed. Furthermore, in order to address the differences between cerebrospinal fluid circulation during wake and sleep we need to work with living animals. Nonetheless, whenever possible, we will replace the in vivo experiments by using primary or organotypic cultures.

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

I considered using immortalised cell lines, cultured neurons, mixed cell cultures and organoids. Literature search of cerebrospinal fluid circulation and lymphatic drainage related terms in Pubmed shows that currently there are no robust non-animal alternatives to study these topics.

Why were they not suitable?

The mechanisms that regulate cerebrospinal fluid circulation can only be fully studied in vivo, in a system where fluid pressure and flow are kept undisturbed. Non-animal models are not able to recapitulate these properties of 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 the proposed project has been calculated based on the animals needed for the experiments we have conducted in our previous project licence.

To reduce the number of animals in the study we will work with cultured and acute brain slices to obtain information about the organisation of vasculature in the brain and how specific cells in the brain react to signals that have been shown to increase or decrease cerebrospinal fluid circulation in vivo. In vivo experiments will be necessary to study the effect of cerebrospinal fluid flow and blood pressure in extracellular trafficking, as well as the dynamical changes of the volume of the extracellular space of the brain between wake and sleep.

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

To estimate the number of animals needed for semi-quantitative description of cerebrospinal fluid circulation and drainage, we have compiled data of experiments done for our previous project license, as procedures were largely the same as for this current

application. For experiments which statistically compare groups of animals (effect of adrenergic modulation on cerebrospinal fluid circulation and effect of solute size on lymphatic drainage), we will use 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?

We are currently developing Monte Carlo simulations to test different working models for the transport of cerebrospinal fluid from the periarterial space into the brain parenchyma and from the parenchyma into perivenous space. For this, we feed our simulations with parameters that we have obtained in the experiments done during our previous license. Our simulations help us optimise number of animals needed to detect differences between treated and untreated samples, combining simulated data with power analysis to calculate required sample sizes. We also refine our calculated sample sizes with small pilot experiments that help us validate simulations and adjust parameters to real world experiments, subsequently using the new simulation codes to adjust animal numbers for the full-scale experiment.

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 organisms to be used will be C57BL/6 mice as well as transgenic Alzheimer's disease mouse models and their corresponding wild type mice controls, since they have a suitably advanced nervous system for investigating the mechanisms we want to address. The majority of experiments in the scientific literature reporting high-resolution studies of cerebrospinal fluid circulation are done in these mice models. In order to maximise the benefit of this project to human health, we also need to extend some of the experiments to transgenic models of Alzheimer's disease and their wild-type counterparts. Transgenic animals will be used from young up to a maximum of 1 year old, timeframe in which the phenotype of the disease is not severe.

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

Protocols 1 and 3 are done under terminal anaesthesia. 90% of animals undergoing Protocol 2 will also be terminally anaesthetised. The need for the 10% of animals which will recover after undergoing Protocol 2 stems from the time needed for the injected substances to diffuse throughout the brain and interact with cells. We cannot use more immature animals because the barriers in the brain are not fully formed and the physiological effects under study will not be fully functional.

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

To minimise the suffering, experiments have been designed in such a way that all stereotaxic surgeries are done under general anaesthesia (e.g. inhalation of isoflurane). Out of these surgeries, 90% will be terminal and the other 10% will allow the animals to recover while they are treated with analgesics. All experiments encompassing cranial windows will be under terminal anaesthesia. In all cases we will use appropriate peri operative care measures as advised by the NVS and surgeries will be carried out under aseptic conditions.

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

The studies will be done in different levels of complexity: cerebrospinal fluid analysis, experiments in cultured cells, in cultured slices, in acute slices, and finally in vivo. These systems are routinely used worldwide in neuroscience laboratories, with well-established protocols that guarantee reproducibility of preparations and are robust enough to be compared across different laboratories. Protocols for dissociated primary cell cultures are commonly used for imaging purposes. Protocols for brain slices are commonly used when connectivity matters, as it is the case in electrophysiology studies and imaging studies that lack meaning in dissociated cells. The protocols for in vivo imaging experiments are well-established and performed in many laboratories worldwide. Additionally, we will use the PREPARE guidelines to design, perform and report our in vivo experiments.

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

Discussing possible improvements to experimental conditions with technical staff and obtaining new ideas for improvement in forums such as LASA and NC3R.

40. Behavioural neuroscience in transgenic zebrafish

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Neuroscience, Motor systems

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?

Under this licence, we want to breed and maintain genetically modified zebrafish lines to provide larvae for experimentation. These experiments will investigate how the brain of this animal produces and modifies movements.

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 ability to move in a controlled and directed manner allows us to look for food, escape predators and dance the boogie-woogie. These movements are produced by precise sequences of activity in our muscles that are started by neurons in our brain and our spinal cord. Even though this process is central to how we move, it is still unclear how the brain does this. We use the larval zebrafish to study this process, as many of the brain structures involved can be found in its brain. We can much more easily study how these brain areas allow the fish to move, as we can use a large number of powerful tools to record and change the activity in specific portions of the brain.

One such brain area, the olivocerebellar system, can be found in all higher vertebrates. It is thought but not yet proven- that it receives information from the brain on what the muscles have been instructed to do, which it then compares with information on what the muscles are actually doing. This allows it to see whether the animal is moving exactly

according to plan. The importance of this has been seen in many patients who develop difficulties when this brain structure is disrupted: poor balance, tremors, and an inability to perform rapid movements. Treatment options for these patients is very limited, involving mostly rehabilitative exercises. While the causes are varied, a greater fundamental understanding of the olivocerebellar system will help future efforts to understand and remedy these conditions.

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

The main outputs of the project will be:

Peer-reviewed publications: in these we will share our findings with the academic community;

Datasets: the data underpinning the publications will be shared freely online;

Transgenic zebrafish: these are powerful tools to address a host of questions in the biomedical research field. They will be shared with any interested party (by supplying the researchers with the embryonic offspring of the relevant adult fish).

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

This project will use cutting-edge tools to investigate fundamental questions in neuroscience. Our findings will benefit the global motor systems and zebrafish research communities, and will help researchers closer to home by providing training and facilities.

Short term benefits:

Motor systems research: Our experiments, which would be extremely challenging in other model organisms, will produce important results, which will be shared in publications and at conferences with data shared online at publication. We will inform researchers working on motor systems globally:

computational biologists can use our insights to update their models on these circuits, and experimental scientists can use our insights to inform their experimental design.

Zebrafish researchers: We will characterise the expression patterns of existing transgenic zebrafish, and develop new ones. These lines are powerful tools in the investigation of the function of specific cell types in neuroscience, developmental biology, and other biological fields. Our results will therefore benefit other zebrafish researchers across the biomedical field.

Medium term:

Further 3Rs agenda: The larval zebrafish is rapidly becoming a premier experimental model for biomedical research. Our project will help strengthen this position further in the UK. We will help achieve the UKRI’s stated aim to advance further improvements in humane animal use in research through partial replacement. For other researchers aiming to do the same, this project will provide an example of a clear pathway towards that goal.

Long term:

Conditions affecting the olivocerebellar system involve poor balance, tremors, and an inability to perform rapid movements. Treatment options are very limited, involving mostly rehabilitative exercises. While the causes for these conditions are varied, a greater

fundamental understanding of the olivocerebellar system will help future efforts to understand and remedy these conditions.

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

We will ensure maximum benefit by sharing all our findings –whether or not they are of direct relevance to the project– informally, at conferences and in publications. New transgenic zebrafish lines will be made freely available to any interested party.

One of the ways UKRI aims to support innovation is by fostering the development of collaborative research programmes. As part of the project, we will collaborate with researchers locally and internationally. This effort will help establish international research links, and will help foster future collaborative projects between institutions.

Our research also lends itself particularly well to be presented to a lay audience, as the output can be presented in striking visualisations. The findings are also relatable and their biomedical importance apparent. We therefore intend to organise activities we think leverage these qualities best, including an annual science festival, and an undergraduate student-led project to develop activities that could be used as part of the primary educational curriculum.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 80,000

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 will use the larval zebrafish as a model organism, which due to its transparency, relatively simple body plan and availability of genetic tools makes it ideal for non-invasive experiments to uncover how the brain generates behaviour. The zebrafish brain also contains many analogous structures to its mammalian counterparts. It is therefore an excellent model system for this project, and ensures we adhere to the UKRI's rule to "Use the simplest possible (or least sentient) species of animal as appropriate to the experiment in question." We will use adults in standard breeding experiments to obtain their offspring which we will use for our experiments.

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

Typically, fish will be bred using standard protocols to obtain their offspring for experimentation or propagation of the zebrafish stock. New zebrafish might be visually screened to check for the presence of genetic markers in pre-feeding animals, or, in rare cases, genetically screened by having tissue samples taken from adults under anaesthesia for analysis. Larval zebrafish (no older than 7 days after fertilisation) will be used in experiments, in which biological processes (typically neural activity) are observed using tools including microscopes and electrodes, and manipulated using tools including drugs to change the processes in the brain. Animals will be immobilised using neuromuscular blockers during the experiments, which will typically take no more than 6 hours.

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

For the most part, we do not expect adverse effects resulting from their genotype, as most animals will only carry mutations/transgenes that have not been reported to cause harm. Our breeding experiments, which may produce animals with homozygous mutations or bring these mutations in a different genetic background, may in very rare cases cause physical abnormalities. These unlikely adverse effects may cause abnormal swimming behaviours and other phenotypes, all of which are expected to be mild. Larvae that display such defects will be humanely killed before the free-feeding stage (5 days post hatching from the egg).

General anaesthesia and fin clipping for genotyping may also produce rare adverse effects, including inflammation and infections.

In rare cases, the mutations/transgenes carried by the zebrafish will affect their fertility rate.

Fish will be checked daily, and all animals will be humanely killed when appropriate.

In rare cases we will need to use zebrafish carrying two copies of a mutation in a receptor that is required for skeletal muscle contraction. These animals are immobile from the point of hatching, and will therefore be humanely killed before they otherwise would have died (after 7 dpf).

Experiments will be performed on larvae (no more than 7dpf) that have been immobilised using neuromuscular blockers, and end in the humane culling of the animal.

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:

Subthreshold (75%)

Mild (5%)

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 interested in how the brain produces behaviour, focusing on how movements are generated, learned, and adjusted. This research question can only be addressed by studying the animal itself. We use the larval zebrafish as a model organism; it is ideal for studying the brain, since its transparency means there is no need for surgery. It is assumed to share many functional similarities with mammals, and is therefore an excellent model system for this project. The use of this relatively simple vertebrate also ensures we adhere to the BBSRC's rule to "Use the simplest possible (or least sentient) species of animal as appropriate to the experiment in question".

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

The FRAME advice has recommendations on how to search for alternatives, starting with the formulation of a focussed research question. Our research question is how the olivocerebellar system receives information from the brain on what the muscles have been instructed to do, which it then compares with information on what the muscles are actually doing. This is one that requires data from the olivocerebellar system itself, which can only be achieved by studying it directly, as there are no publicly available datasets that can be studies. Alternatives would have been:

Computational modelling: this can be useful to generate hypotheses about how the brain operates, or to test interpretations of experimental data

Cell cultures: networks of neurons grown in a dish can be used to study how these cells might interact.

Why were they not suitable?

We will use the larval zebrafish to chart its neural activity and discover which areas in the brain are involved in generating movements. Computational modelling cannot reveal this, as there is currently insufficient data to generate accurate models

Cultured neurons are unlikely to recapitulate the organisation of the brain and spinal cord of the larval zebrafish. Using this technique is therefore unlikely to produce insights into how the neural circuitry in the brain and spinal cord interact to produce behaviour

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 estimated how many animals were required in our experiments using standard calculations. Using common assumptions about the variability of our data, we estimated we needed more than, or equal to, 9 animals per experiment. In some experiments, where we compare a biological process before and after a manipulation, we need at least 15 animals per experiment to ensure we have confidence in our findings.

We expect to be performing 3-7 full experiments per week. If we perform an equal number of descriptive and manipulation experiments, we will require 60 pre-feeding animals per week. For the 5year duration of the project that makes ~15,000 animals. From experience,

we know that ~20% of all offspring can be used in experiments. This is because each cross typically results in a large number of embryos, most of which will not be the right genotype (these will be killed before they reach 5dpf using an SK1 method), and some of which will die of natural causes. This suggests a total number of offspring required of ~75,000. In order to obtain these numbers, we require ~1,500 crosses. Assuming 15 different genotypes will be used for the project, this can be achieved with 2 holding tanks per genotype, with ~25 fish each. Assuming a mean lifespan of ~1 year per fish, this will require 3,750 adult fish, for a total of ~80,000 animals (75,000 + 3,750).

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

Advice was sought from previous PPL applicants. Furthermore, the NC3Rs' Experimental Design Assistant was consulted, and dedicated software was used to do power and sample size estimations (G*Power).

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

For our breeding and maintenance procedures, we will carefully adjust the number of animals we use according to our requirements and the constraints posed by the animals. For instance, the number of breeding animals for each mating will be tailored to the number of offspring we require from them for each experiment; the fertility of each particular stock of zebrafish will also be taken into account (i.e., a stock that has a low fertility rate will need to have its numbers increased). This means we will not use more than the required animals for each experiment; nor we will use too few, which would not allow us to do statistically rigorous work. Furthermore, we have done power analyses to estimate required sample sizes for our experiments on larval zebrafish.

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 aims to understand how the brain produces behaviour; the use of animals therefore cannot be avoided. We will use the larval zebrafish as a model organism, which due to its transparency makes it ideal for non-invasive imaging. Its brain is assumed to share many functional similarities with

mammalian homologues. It is an excellent model system for this project, and ensures we adhere to the UKRI’s rule to “Use the simplest possible (or least sentient) species of animal as appropriate to the experiment in question.”

For our experiments we will use well-established protocols to breed and maintain transgenic zebrafish and prepare them for experiments, ensuring minimal harm and distress. GAA passports for each line are established on a local level, frequently updated with new information, and include details relating to changes to the phenotype, breeding

performance, colony management, mortality rates etc. A list of the GAAs kept at the establishment plus the GAA passports are held with the PPL, and will be provided to the Home Office Inspector on request.

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

In order to generate transgenic larval zebrafish, we need to use animals that have reached sexual maturity. The use of adult zebrafish is therefore required. Furthermore, the experiments described in protocol 4 use the earliest life stages that produce behaviour similar to adult zebrafish, namely between 5 and 7 dpf.

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

When using anaesthetics, type and depth of anaesthesia have been carefully selected in consultation with the NVS. Following fin clipping in >5dpf fish peri-operative analgesia will be provided; agents will be administered as agreed in advance with the NVS. The procedure will be carried out using sterile equipment, and the site and amount of tissue removal for genotyping will be such that there is no compromise to normal swimming. Infections can result from fin clipping (<1%) or from damage to scales or loss of mucous surface from swabbing. Any fish exhibiting any abnormal behaviour or signs associated with infection will be humanely killed.

When we create GA founders, embryos will be assessed for morphological phenotypes before the stage of independent feeding using standard light and fluorescence microscopy, and any showing morphological abnormality not required for the scientific purpose would be humanely killed. Some genetic alterations may result in a harmful phenotype during post-hatching development evidenced as failure of larvae to inflate the swim bladder, difficulty swimming, altered morphology, failure to feed or breathing difficulties. If fish exhibit any of these adverse effects they will be killed immediately by a humane method. On occasion (<5%) late onset mutations in more mature stages may lead to lines of fish with mild lordosis (defined as less than 20% curvature of the spine), poor growth/body condition or difficulty swimming. Any fish showing harmful phenotypes will be humanely killed as soon as the phenotype is visible. During our experiments on larval zebrafish we will monitor the heart rate and blood circulation of experimental animals, and will check animals for signs of injury to ensure there are no signs of distress or harm. Any fish showing these signs will be humanely killed as soon as possible.

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

We use well-established protocols to breed and maintain transgenic zebrafish, ensuring minimal harm and distress.

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

The NC3Rs is the pre-eminent UK-based scientific organisation dedicated to replacing, refining and reducing the use of animals in research and testing. We will monitor the NC3Rs' output to stay aware of the latest developments in animal welfare, and implement these whenever possible.

41. Developing Tissue Engineered Intestinal Grafts to treat inflammatory bowel disease

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

Key words

Inflammatory bowel disease, Colitis, Stem cells, Regenerative medicine

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?

Our aim is to establish whether intestinal tissue grown from stem cells can be successfully implanted into the colon. If it can be, we want to find out whether this new tissue could act as a new therapy to aid healing in when the bowel is inflamed.

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?

Inflammatory bowel disease (IBD) is relatively common, long-lasting and usually begins in young adulthood, meaning patients have a high disease burden throughout their lives.

This results in a poor quality of life - owing to long-lasting pain, diarrhoea and poor nutrition - as well as placing a high cost on the health service.

The disease has three main mechanisms 1) impaired “leaky” barrier in the bowel to microorganisms, 2) movement of microorganisms into the bowel wall, 3) sustained inflammation. These factors form a vicious circle, making the disease difficult to treat.

Most current therapies act by targeting one of these mechanisms - through suppressing inflammation.

For many patients this does not work, however. In around 4 out of 10 patients, these treatments are not fully effective and surgery is required, often to remove large sections of diseased bowel tissue.

We therefore urgently need to develop new treatment approaches. We want to do this by targeting other mechanisms which cause IBD - specifically to improve healing of the bowel lining so that it can act as an effective barrier to the bowel contents and infectious agents. This could be used in combination with other approaches (such as current treatments) to reduce the number of people who experience severe disease and who need surgery.

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

Further funding: the work outlined here will provide pre-clinical data to support the development of this technology as a clinical product. We anticipate using this to support an application for the next steps in clinical translation.

The pre-clinical data we would expect to gather from this work are directed at the following questions:

how well tissue grafts can be transplanted;

what we might need to do improve the transplantation procedure (such as different cell types,supporting materials);

how well the tissue grafts aid healing in inflamed bowel.

Specific datasets include histological data from analysis of the tissue structure and molecular profiling data. All data will be shared with the research community on publication in line with specific journal requirements and the latest biomedical data-sharing practice.

Publications: we anticipate publishing this work in a scientific journal, at this stage this would be to demonstrate 'proof-of-concept' data that this approach is feasible.

Information: the feasibility of the approaches undertaken in this study will be useful for planning further studies in future, by informing us on the design of future experiments.

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

Benefits can be divided into those for the academic community, through deriving knowledge, and those for patients with IBD.

Our aim is to investigate the potential for regenerative medicine technology based on stem cells to be used as a treatment for IBD. The benefit of the treatment to patients is unlikely to occur during the timeframe of the project, but it would provide data to give us and regulators confidence to move to the next step of in-human testing.

Understanding how stem cell-derived intestinal tissue and advanced materials behave in an appropriate mouse model is very valuable knowledge in itself, which will benefit the wider academic fields of regenerative medicine, stem cell biology and materials science.This could drive investigation of other treatments in IBD. In addition, understanding tissue transplantation requirements could lead to scaling up the technology for other bowel diseases where there is tissue loss due to surgery (for example, cancer,

diverticular disease, ischaemic colitis) with larger sections of bowel being able to be created as a result of our findings.

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

Collaboration: we will use our existing network of clinical, tissue engineering and materials science collaborators to drive this work forward by changing various design factors of the tissue graft, to inform further development as a clinical product.

Dissemination: we will publish the work in high-impact peer-reviewed journals (with open access). We will present the work at both clinical and tissue engineering forums. We will share our findings transparently (allowing full access to data) so that others can learn from both successful and unsuccessful approaches.

Species and numbers of animals expected to be used

- Mice: 80

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 cannot pilot this technology in humans as it is extremely novel and therefore we do not have sufficient data to be confident that it would 1) be effective in treating IBD and 2) be safe as a treatment.

To be able to begin the process of clinical translation, we therefore need to adopt appropriate preclinical models to understand how this technology behaves in the body, to decide whether it is worthwhile investing in, and developing this technology further and how we could refine our approach as we develop this as a clinical product. It would also be unethical to attempt to re-create the disease in humans or attempt to treat humans suffering from the condition, with an untested therapy, without understanding how it behaves in the body .

For clinical translation, we need to use an animal which has a similar structure to humans and in which a disease state similar to human IBD can be induced. In addition, we need to use an animal which can be immunocompromised to allow human tissue to be implanted. Specially bred immunodeficient mice fulfil all of these criteria and represent the lowest species in which all these criteria are met.

We intend to use adult mice as the well-established model of inflammation uses this life stage and in addition the colon is fully developed and thus suitable for surgery and also to recreate how the human colon would behave.

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

The mice will undergo a general anaesthetic and will then have their abdomen opened using a surgical incision.

The intestinal tissue graft will be placed in a small pocket created inside the wall of the large bowel. This will be covered with a special mesh which aids healing and prevents scar

formation. The cut in the abdomen will be then be stitched. The mice will recover from the anaesthetic with pain relief. The surgical procedure should last around 30 minutes.

The mice will monitored for any signs of distress and mice will be kept alive for up to four weeks and then humanely killed.

In some mice (50%), inflammation will be induced beforehand by feeding them a chemical in their drinking water. This will take place over the course of a week before the graft is implanted. In some mice in this group (50%), no tissue graft will be implanted. The purpose of this is to ensure the tissue graft is improving healing rather than the surgical procedure alone.

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

The surgical implantation procedure will involve forming a small pouch within the bowel wall and inserting the tissue graft, which will covered by surgical mesh. After implantation takes place, the abdomen will be sewn for closure. The operation will be performed under general anaesthesia and pain relief will be given during recovery from the operation. The expected severity is moderate.

Colitis will be induced by feeding dextran sodium sulphate (DSS) to mice in drinking-water for 1 week. Mice may experience weight loss, diarrhoea or dehydration, which will be monitored. Mice will be given therapy for rehydration if needed. The expected severity is moderate.

Potential complications (<1%) include intestinal obstruction, perforation, infection or persistent pain. If there are any signs of these (including pain, altered bowel habit, swelling around the surgical site, altered behavioural signs), the animal 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)?

All mice will experience no more than 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?

We have performed extensive laboratory-based testing of cells before planning this project and have also performed previous animal work in the more accessible skin site. As we intend to develop this technology as a clinical therapy for IBD, we need to know how the generated tissue will behave when implanted into the bowel, and particularly the inflamed bowel. In the bowel specifically, we need to know the effects of the bowel contents, the movements of the bowel and the other cell populations which will interact with the

implanted tissue. There is no laboratory-based model which can recreate these factors, and therefore replicate the complex environment of the animal/bowel.

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

We use laboratory-based models and molecular analysis of cell behaviour and maturation to refine our techniques in parallel (such as varying chemicals and materials) and only test the most promising combinations in the animal, once fully optimised in the laboratory.

Specifically, we use molecular techniques to profile the expression of certain genes that can inform us how well our cell populations resemble normal colon cells after being derived from stem cells (which form the developing embryo). We also use these techniques to confirm that no very early stem cells remain (as these could form unwanted tissues in the body).

Why were they not suitable?

As we intend to use this technology as a clinical therapy, we need to understand how the tissue will behave in the correct host environment. While molecular techniques and growing cells outside the body can give us a lot of information, they cannot tell us how cells will respond to their real life environment. There is no laboratory-based model which can adequately account for all these factors and therefore no replacement for placing the tissues into the bowel in a suitable animal 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?

We will perform pilot experiments first with a small number of animals (up to 5 in each step) to understand graft efficiency and variability, and to optimise the procedure. Using data from these pilot experiments, we will then use the most efficient experimental design so as to gain the maximum information from the fewest animals in the subsequent studies, where we expect to use no more than 15 per group Potential materials will first be tested rigorously in vitro (in the laboratory) to ensure compatibility with cell populations before proceeding to animal studies.

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

After completing pilot studies, we will use the NC3R's Experimental Design Assistant to estimate the minimum number of animals required to gain sufficient power in each group.

All experiments will adhere to the PREPARE and ARRIVE Guidelines on design and reporting of animal experiments, and good principles of experimental design, including the use of the NC3R’s Experimental Design Assistant, which, after completing pilot studies, will be employed to ensure sufficient animal numbers and group sizes will be used to adequately test the hypothesis. We will ensure that we use the minimum number of animals required to answer the scientific question by performing power calculations.

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, wherever possible, carry out growth optimisation studies on the grafts in our lab, prior to planning the animal studies.

Our implantation model in previous experiments has had a high success rate for transplantation which reduces the number of mice required. We will be optimising the surgical techniques using mouse cadavers after termination of other studies.

When testing new materials we will use pilot studies to understand variability to enable us to design studies with appropriate numbers to demonstrate differences between groups.

As we are only interested in the gut tissue, we will make any other organs available to other researchers and projects .

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.

Adult mice will be used as we require a species with an appropriate mammalian body system, and it must also be able to generate a sufficient amount of tissue. They represent a good choice of species for implanting (xenografting) human tissue, as numerous well-established immunodeficient mouse strains have been developed which allow for human xenografts to be accepted. Mice are the lowest species in which a genetic mutation has produced the required immunodeficient status. In addition mice are excellent models of IBD which can be readily induced by introducing specific chemicals into water or feed.

The mice will have a general anaesthetic and then surgery to implant a small tissue graft into the bowel. This was chosen as the technique is relatively straightforward and allows the easiest access to the bowel. The implantation will result in a submucosal rather than intramucosal tissue graft. At this stage, we want to understand i) whether the iPSC-based tissue graft is engraftable into the colon and ii) how the graft will behave in the environment of the colon. Undertaking an intramucosal graft procedure would add considerable complexity to the study and likely increase the risk of adverse effects. At this stage in the project, we want to understand whether the graft can be implanted into the colon and thus the proposed, relatively simple, model will enable us to ensure this question.

The use of invasive surgery is needed to access the colon. Performing a colonoscopy was considered but this would be technically challenging. This would limit the size of tissue graft we could test to a very small area which would not be as meaningful in its results. In addition the technical difficulty of a colonoscopic approach would increase the risk of adverse effects such as bowel perforation.

Animals will be carefully monitored to ensure they are not in distress and will be given appropriate pain control after the operation. The study uses a relatively short timeframe

which we know from previous experiments under the skin is sufficient for the aims of the experiments.

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

The colitis (bowel inflammation) model is established in adult mice. In addition, the immature bowel is smaller than that of an adult which would compromise the experimental aims (by reducing the size of graft which can be implanted) and could also increase the risk of adverse effects (as the smaller bowel would be more difficult to operate on).

Adult mammals are required as they adequately replicate human tissues and body systems, which we need to ensure the implanted tissues are subject to similar conditions as would experienced when implanted into the human colon. Mice are the lowest sentient species in which tissues will grow in this comparative manner.

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

Within the DSS-colitis model, the dose of DSS will be optimised at a small scale. It has been found that there is some variability in response to DSS in animals housed in different facilities related to the local environmental conditions. One factor which has been found to be especially important is whether animals are housed in ultra-clean conditions, with a lower dose of DSS generally required in animals in such conditions. As the animals to be used in this study are immunosuppressed and thus kept in strict aseptic housing facilities, it is anticipated that a dose at the lower end of the 2-5% w/v scale will be required. The process of optimisation will be to use the lowest dose within the range to demonstrate sufficient disease activity, only increasing the dose if necessary to acheive adequate disease activity.

Animals will be monitored by trained technical staff daily and they will be weighed and bowel habits assessed using standardised scoring systems. The daily staff also have constant access to others,e.g. the Named Veterinary Surgeon, the Named Animal Care and Welfare Officer who they can consult for advice . Monitoring will be particularly increased during post-operative recovery for the first 24 hours to observe for signs of adverse effects. A number of disease activity characteristics (consistency of stool, rectal bleeding, weight loss, rectal prolapse, anaemia) will be specifically monitored and scored using a standardised system

The intended scientific endpoint is the study end (4 weeks), however, animals with either higher disease activity (over a specified score threshold) or with specific highly scoring individual symptoms (rectal bleeding, anaemia, weight loss, prolapse) will be humanely killed as soon as these symptoms reach a critical score i.e before the end of the study.

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

NCRI Guidelines for the Welfare and Use of Animals in Cancer Research

LASA Best Practice Guidelines

Norecopa PREPARE Guidelines

ISSCR 2021 Guidelines for Stem Cell Research and Clinical Translation

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?

Internal sharing of important updates (users mailgroup).

Subscription to NC3R's newsletter.

Regular contact with AWERB and Named Persons, including the Named Information Officer, and NC3Rs.

Attendance at conferences/meetings relevant to the project, 3Rs and animal use in research.

42. Effects of pollution and other environmental stressors on amphibians

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

Amphibians, Biodiversity, Ecotoxicology, Stressor, Behaviour

Animal types	Life stages
Lissotriton vulgaris	Embryo and egg, Neonate, Juvenile
Rana (Rana temporaria and Rana pipiens)	Embryo and egg, Neonate, Juvenile
Bufo bufo	Embryo and egg, Neonate, Juvenile
Lissotriton helveticus	Embryo and egg, 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 aim of this project is to investigate the effects of pollutants, their mixtures, and other ecologically relevant environmental stressors on amphibians in order to protect wild populations.

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?

Amphibians are the fastest declining vertebrate group, with 40% of described species threatened with extinction (in the UK, 3 out of 7 native species are declining/threatened – 2 with full legal protection [natterjack toads, Epidalea calamita, great crested newt, Triturus cristatus]). Another 15% are classed as "data deficient", so the number of threatened species may be even higher. Chemicals and other environmental stressors (e.g. climate change induced freshwater salinisation/ heatwave events, invasive species) are known to negatively impact biodiversity, both alone, and when present simultaneously or sequentially in the same habitat. The aims of this work, therefore, are to delineate the effects of environmental stressors on amphibians using a range of environmentally relevant exposure designs. In addition to scientific aims, this research could directly impact conservation strategies or policy.

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

In this project, we will generate new knowledge for harm caused by chemicals and other environmental stressors. The ultimate aim of this work is to benefit wild amphibian populations by contributing understanding surrounding causes of declines and extinctions. Therefore, the main output is increased understanding of role of stressors in amphibian decline and quantification of multi-stressor impacts. Methods developed throughout the project, including advances in multiple stressor theoretical approaches, have wider applicability beyond amphibian ecotoxicology.

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

The output of this work is to gain understanding for the effects of environmental stressors – including co-occurring stressors – on amphibians; ultimately contributing to the protection of amphibian biodiversity. Amphibians form critical components of freshwater and terrestrial ecosystems. Benefits to the protection of amphibian biodiversity provide benefits to whole ecosystems and to human health and wellbeing. The data produced from this work will be highly valuable to academic researchers studying ecotoxicology and environmental science, governmental bodies responsible for environmental regulation and policy, and conservation organizations working to protect biodiversity. This research will offer these stakeholders critical insights to guide conservation actions, regulatory frameworks, and ecosystem management practices. Further, amphibian biodiversity contributes to ecosystem services that support human health, such as controlling insect populations that spread diseases and maintaining water quality through natural filtration processes. As amphibians decline, these ecosystem functions deteriorate, potentially leading to increased incidences of waterborne and vector-borne diseases

It is widely recognised that pollutants and other environmental stressors are negatively impacting terrestrial, marine and freshwater biota, however, little is understood about how these threatening processes impact amphibians. Gaining understanding of this question is important for protecting ecosystems from harm. Uniquely amongst vertebrates, amphibians traverse freshwater and terrestrial environments, and thus have the propensity to be disproportionately impacted by diverse environmental stressors. To date, the vast majority of research into the effects of environmental stressors amongst freshwater biota are carried out with fish, and there is a need to understand effects on amphibians more fully. This project is focused on investigations into the effects of pollutants and other environmental stressors on amphibians, both singly and in combination. The combination of environmental stressors encompasses a growing area of research, in recognition that

environmental stressors are rarely present in the environment singly, and instead can cause joint toxicity to organisms when present in combinations.

The scope of this work encompasses environmentally relevant exposure scenarios, in order to produce data outcomes with high relevance to wild populations. Data will be made available to the wider scientific community through normal routes, such as open data repositories (as required for UKRI funded research) and associated publications. In addition, a focus on taking non-destructive measurements, such as length and body mass measurements and analyses of behavioural endpoints, facilitates method development related to the reduction and refinement of the use of animals in research.

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

My research group works closely with governmental agencies and charities focused on the protection of amphibians and their habitats within the UK. Due to long-standing and ongoing collaborations with these groups, project updates will continue to be disseminated through steering group meetings, established networks and via conference attendance/presentations. In addition to academic publications and conference presentations, the research findings will be disseminated via public outreach events, webinars, and social media platforms to engage a broader audience, including policymakers and the general public. This multi-channel dissemination approach ensures that the project's outputs reach diverse stakeholders, from conservation scientists to community conservation groups. Unsuccessful approaches are also discussed within these interactions, so that refinement of approaches, including integration/consideration of ‘non-significant’ effects, are fully considered and applied to future research endeavours.

Species and numbers of animals expected to be used

Rana (Rana temporaria and Rana pipiens): 6000 Other amphibians:

Bufo bufo: 6000

Lissotriton helveticus: 1500

Lissotriton vulgaris: 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.

The amphibian species used in this project are those native to the UK, including anuran (common frog [Rana temporaria], common toad [Bufo bufo]) and caudate (palmate newt [Lissotriton helveticus], smooth newt [Lissotriton vulgaris]) species. Amphibians are declining in the UK, and in order to delineate reasons for this decline - driven by localised extinctions - it is essential to investigate for the impacts of environmental stressors. Due to the taxonomic diversity of UK amphibian species, they are likely to respond differently to environmental stressors, and as yet, we do not have good evidence that allows read-across between species for their responses to environmental stressors. Natterjack toads (Epidalea calamita) and great crested newts (Triturus cristatus) are not included in this

research programme due to the high level of legal protection, as well as limited distribution, for these two species. For the remaining native UK species, it is important that these are collected from the wild, so that populations with different exposure histories (and resilience) to environmental stressors can be compared - allowing the delineation of appropriate mitigation measures to contribute to the prevention of localised extinctions. Early life stage exposures to pollutants and other environmental stressors are known to impact life history traits throughout the animals life (such as survival, growth and reproduction). Therefore, it is important to carry out exposures during aquatic larval exposures (embryos, larvae) and investigate effects on both early and later (i.e. juvenile/sub-adult) life stages.

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

Typically, amphibians will be exposed to combinations of environmental stressors or pollutant mixtures from early larval stages until the completion of metamorphosis/post-metamorphic juveniles (or, to early juvenile stages in newt species, which do not undergo metamorphosis). These exposures will typically last 10-14 weeks, and in some cases, effects will be assessed up to 14 weeks post-exposure cessation. Each individual is expected to undergo one primary exposure procedure, with regular behavioural monitoring conducted daily or bi-weekly, depending on the specific protocol. Although these exposures are hypothesised to cause harm, exposure levels for the environmental stressors are designed to be environmentally relevant, and thus encompass low and non-lethal levels. Where available, data will be obtained from the peer-review and grey literature and combined the expertise of the PI in the determination of appropriate exposure levels. Exposures are not expected to result in mortality of the individuals beyond levels observed in control (unexposed) individuals. Exposed amphibians will be carefully monitored (detailed below) for evidence of harm and humanely euthanised according to pre-defined criteria (detailed below).

In addition to the controlled exposures detailed above, field-based experiments will also take place. Field-based experiments are essential for understanding how these responses manifest in real-world scenarios, where multiple environmental factors interact. Together, the complementary field/laboratory approaches will provide a comprehensive understanding of amphibian responses to environmental stressors.

Field studies will comprise caging embryos in their native habitats as well as transplanting embryos from reference/clean to test/polluted environments. The purpose of these experiments is to investigate the effects of pollutants and other environmental stressors on amphibians in real-world exposure scenarios. Amphibians will be regularly monitored for adverse health outcomes as described above, however, with a lower frequency than for the controlled exposures due to practical limitations of travelling between field sites. The interval duration for monitoring of field-based experiments will range between 7-21 days, depending on the specifics of field site characteristics (i.e. remote, permanent/deep water bodies will be visited less often than more public/visible, shallow water bodies).

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

Sub-lethal adverse effects on the exposed organisms are implicit with the experimental design of the project, but these are expected to be minimal due to the low levels of pollutants and other environmental stressor exposure levels. Potential sub-lethal effects may include mild changes in swimming behaviour, reduced feeding rates, or slight developmental delays. These are expected to be temporary and reversible as exposure

levels are carefully designed to avoid more than moderate harm, and will be closely monitored to mitigate any lasting impacts. However, as exposures will comprise novel components, effects on amphibians are as yet unknown. Due to the general lack of data on amphibians, any existing data for effects on amphibians will be combined with exposure effect data for other aquatic vertebrates (i.e. fish). Using these data sources, selected exposure levels will be designed to cause sub-lethal effects on the amphibians used in this project. Certain sub-lethal endpoints will be used to identify any animals in distress (oedema, loss of equilibrium/swimming ability, floating on water surface). During exposures, animals will be monitored on a daily basis by a trained staff member to ensure timely detection of distress. Previous experience of carrying out amphibian exposures with a large range of species have shown that any larval amphibians showing these signs of distress are very unlikely to recover and therefore they will be immediately removed from the experiment and euthanised by a Schedule 1 method. Thus the duration of these effects would be minimal.

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)?

As amphibians will be exposed to pollutants and other environmental stressors at low levels, sub-lethal effects on physiology and behaviour are expected. Therefore, the experiments carried out under this project licence are classed as moderate, with the exception of animals in the control (unexposed group). It is therefore expected that for 90% of the animals used in these experiments they will experience a severity of 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?

Due to the paucity of data for effects of environmental stressors on amphibians, there are currently no in vitro nor computer-based alternatives for this type of research and there are no plans to develop these within the project. Due to the poor current state of knowledge for sub-lethal effects of stressors on amphibians, along with a lack of appropriate modelling approaches for effects of co-occurring stressors on biota, developing these within this project is out-of-scope. However, data collected during this project would eventually have the potential to be applied to such approaches. Within this project, we will also be specifically focused on non-destructive measures, such as behavioural observations, which will routinely be included within experimental designs to address experimental objectives – as alterations in behaviour can also be considered to result in negative outcomes for individuals and populations.

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

There are no in vitro or computer-based alternatives for this type of research.

Why were they not suitable?

There are no in vitro or computer-based alternatives for this type of 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?

For all experiments associated with this project, experimental design is based on statistical planning. Careful consideration of existing literature, including our own unpublished and published data is used to inform experimental design. Tank replication has also been considered as there may be variability caused by different social groups within tanks. This allows us to use the minimum number of individuals while ensuring statistical robustness. Frogs (Rana temporaria) and toads (Bufo bufo) have been selected as the primary species for study due to their higher sensitivity to environmental stressors, as documented in both published and unpublished studies. In addition, there is a larger amount of previous research on these groups, thereby better informing the design and exposure levels for laboratory based experiments. Their larger population sizes in the wild and well-documented responses to pollutants make them ideal models for investigating the effects of stressors. Newt species will be included in smaller numbers, as they have different ecological niches and may exhibit different responses, requiring fewer individuals for comparative purposes.

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 required for each experiment was determined through statistical power calculations, ensuring that sample sizes are large enough to detect meaningful differences while avoiding the use of excessive numbers of animals. This ensures a balance between scientific robustness and ethical responsibility. Further, co-design of experiments with leading ecotoxicologists that form part of the PI's wider network ensures statistical approaches are appropriate and robust, and occur both during experimental design and of the final experimental design prior to the start of experiments. Experimental designs and associated statistical analyses are always critically appraised by the team involved with the research prior to the start of each experiment. This input to data analysis approaches allows us to continuously refine our experimental designs and to minimise the number of amphibians being used.

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

Where data for amphibians or fish are too scarce to be able to make reasonable predictions on the toxicity of pollutants or other environmental stressors, small scale pilot studies (75 % smaller than full experiments) will be used to produce dose-response data

(anticipated severity: moderate; i.e. sublethal effects). These data will be used to effectively select exposure levels in full scale experiments that are not expected to result in lethality. All data produced will be analysed to the maximum extent possible, by incorporating effects on physiological endpoints into ecological models to predict potential effects of exposures on populations (i.e. smaller pilot studies and larger full-scale experiments). In addition, the number of different tissues extracted from exposed organisms and how they are analysed will be maximised via the PI's national and international networks with other researchers working in the area of amphibian ecotoxicology. The sharing of tissues will act to reduce the total number of animals used in experiments by maximising the data outputs from each experiment (none of the species on this license are subject to tissue transfer restrictions).

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.

Exposure of amphibian species followed by humane euthanasia. By consulting the literature and drawing on the experience and the PI's in-depth knowledge/experience in the training of personnel, will result in minimal suffering of the organisms. The PI shall ensure that the appropriate level of supervision is provided for all personal licensees carrying out regulated procedures under the authority of this licence. The PI will be on site more frequently to ensure competence of staff in the early stages of experiments. Pilot studies using a smaller number of animals will be used for pollutants and other environmental stressors with unknown toxicity.

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

In order to assess the potential harm of environmental stressors to wild amphibian populations, it is necessary to conduct experiments on larval life stages and assess effects up to juvenile stages. This is because there is a lack of data on how early-life stage data can be used to predict effects on later life stages. As part of this work we will use data collected on early life stages and how this relates to later life stages, to inform best practice going forward regarding how early-life stage exposure relates to later health outcomes.

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

Procedures will be refined as appropriate upon regular reviews of the experimental outcomes, with the aim of reducing animal numbers, exposure duration/levels. All students and staff working on associated amphibian exposure projects will be fully trained to recognise harm in the animals and euthanised as appropriate. Distress is determined by the observation of oedema, loss of equilibrium/swimming ability or floating on water surface. During exposures, animals will be monitored daily to ensure timely detection of

distress. From the PI's extensive experience of carrying out amphibian exposures with a large range of species, any larval amphibians showing these signs of distress are unable to recover and therefore they will be immediately removed from the experiment and euthanised by a Schedule 1 method.

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

Apart from OECD guidelines for exposures with Xenopus spp., these are lacking for amphibians.

However, exposure conditions for UK native amphibian species generally follow those used for

Xenopus spp. The PI has conducted exposure experiments with a wide range of amphibians, including Ranids, Bufonids and Xenopus. They will continue to adapt OECD guidelines for Xenopus as needed for the UK native species.

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

By regularly surveying the literature, the PI will be able to keep abreast of any developments within the 3R’s regarding amphibian exposure experiments. In addition, the PI will regularly liaise with NTCOs and the NIO at the Establishment. All staff involved with amphibian exposures will receive specific training from the PI on how to identify suffering and distress in larval/juvenile amphibians. New data produced as part of this project will be used to update guidelines as appropriate. In addition, via her collaborations with researchers carrying out exposures with fish, she can look to apply advances in fish toxicology to the tadpole stages as appropriate.

43. Phenotypic programming across the lifespan in birds: understanding ecological and welfare perspectives

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Development, Environmental challenges, Welfare, Birds, Neuroendocrinology

Animal types	Life stages
Domestic fowl (Gallus gallus domesticus)	Embryo and egg, Neonate, Juvenile, Adult
zebra finch (Taeniopygia gutatta)	Embryo and egg, Neonate, Juvenile, Adult
Pheasant (Phasianus colchicus)	Embryo and egg, Neonate, Juvenile, Adult
Guinea fowl (Numida meleagris)	Embryo and egg, Neonate, Juvenile, Adult
Japanese quail (Coturnix japonica)	Embryo and egg, Neonate, Juvenile, Adult
Lesser black backed gull (Larus fuscus)	Embryo and egg, Neonate, Juvenile, Adult
Herring gull (Larus argentatus),	Embryo and egg, Neonate, Juvenile, Adult
Kittiwake (Rissa tridactyla)	Embryo and egg, Neonate, Juvenile, Adult
Fulmar (Fulmarus glacialis).	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?

To determine how environmental conditions during different life stages can influence health and wellbeing in the short and long-term in a range of bird species.

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?

Animals live in a changing world, especially those living in the wild, where the potential impacts of climate change will be felt over the next few decades. Even in captivity animals experience change and it is important that we ensure that we provide environments that maximise the wellbeing of the animals. This research therefore has two important strands, the welfare of captive animals and the conservation of free-living animals.

Importantly the work we propose will investigate how early life experiences interact with adult experiences to influence the ability of animals to cope with change in a range of environments. We will also perform experiments that allow us to determine if the effects we see are beneficial or costly to the animal in the longer term. Often researchers only look at the welfare or wellbeing of an animal at one stage of its life - normally in adulthood. However, the early stages of life are vital for setting up all the internal systems the animal needs to cope with change in the future. So, by studying how animals develop and cope in these different environments, we will be able to create knowledge that will not only tell us what the effects of a changing environment are on animals, but how we can reduce the impacts on human managed species and animals in the wild. This knowledge is critical in developing practical ways in which we can increase animal welfare. Without understanding the importance of developmental conditions in shaping health and wellbeing we miss the opportunity to create effective interventions to help animals in a changing world.

The study of animals is critical to the project success. These studies would not be relevant in humans, as the focus of the work is on animal health at the organismal level as well as their behaviour.

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 way in which environmental conditions program individual health and wellbeing.

We will produce data firstly that show how living in different environments at different life stages can alter the way in which animals behave and cope with challenges across the lifespan, and also seek to determine which environmental changes are the most influential on the animal. Secondly, we will be able to pinpoint which physiological traits that underpin these effects and hopefully feed into interventions in captivity and the wild that can ameliorate any detrimental effects that we see. In these experiments we will produce many types of data (e.g. behavioural, anatomical, physiological, gene expression patterns) that will refine our understanding of how animals can be impacted by changing environments. The main outputs will be academic papers that will span all the data types we collect, and we aim to collate data together to enable us to tell the whole story, so we may bring together data from different experiments to allow us to do that and create the most useful publications for the field and for future intervention design. We will also publicise the findings of our experiments 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?

Neuroscientists have become increasingly interested in how the brain changes over the course of the ageing process and into senescence, as this can be highly relevant to several human pathologies. However, there are few studies that have tracked individuals from known developmental backgrounds through adult life to explore the interactions between environmental conditions at different life cycle stages.

The developmental origin of health and disease is an important focus for a range of research areas, including those working in basic biomedical and clinical sciences and agricultural research. This work will provide a novel opportunity to gain an understanding of not only how early life can program adult susceptibility to disease pathologies, but also how the environment experienced during adult life can interact with the developmental experiences to shape the potential for predisposition for a range of disorders, including stress related disorders and cognitive decline.

Animal welfare scientists have increasingly adopted behavioural and physiological measures, such as cognitive performance and stress reactivity, for assessing the wellbeing of animals. This project will add to their understanding of how such traits and several others relate to both adult and developmental environmental conditions. This could facilitate direct changes to animal husbandry protocols that would feed into our commitment to reduce animal numbers and refine our techniques to ensure high welfare conditions for all animals both within scientific research, but also in commercial and production settings too.

Finally, our work will also look at how environmental conditions, both natural and anthropogenic (e.g. exposure to environmental contamination) can impact on the health of bird species so our work has high relevance to conservation organisations and agencies working to understand population changes in our changing world.

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 biological 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 School of Psychology & Neuroscience and also with the wider St Andrews research community through the Institute for Behavioural and Neural Sciences (IBANS) and the Coastal Resources Management Group. The applicant also collaborates with colleagues within Computer Science to generate new tools for refinement of monitoring and behavioural assessment.

Field/interdisciplinary levels: we attend specialist conferences across a range of disciplines, organised by societies such as British Neuroscience Association, Association of the Study of Animal Behaviour, Developmental Origins of Health and Disease, Seabird Group to disseminate findings and develop new collaborations. We also collaborate with NGOs and government agencies to carryout work and ensure our data is accessible to important stakeholders for applied use, such as Marine Scotland, British Trust for Ornithology, Animal Welfare Research Network, Nature Scot and the Animal and Plant Health Authority. 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 though larger Science Festivals.

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

Domestic fowl (Gallus gallus domesticus): maximum 500

Other birds:

Guinea fowl (Numida meleagris): maximum 500 zebra finch (Taeniopygia gutatta): maximum 300 Japanese quail (Coturnix japonica): maximum 500 Lesser black backed gull (Larus fuscus): maximum 250

Herring gull (Larus argentatus),: maximum 250

Kittiwake (Rissa tridactyla): maximum 250

Fulmar (Fulmarus glacialis).: maximum 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.

We will use birds, such as the chicken and zebra finch, as a model in this research as we know a great deal about their developmental trajectories, ageing rates and physiology and behaviour. An important feature of avian species is that they overcome a major constraint of mammalian studies that look at the long-term effects of developmental conditions, namely the direct link between the mother and developing animal during both gestation and lactation. This prolonged link, limits the ability to determine the exact conditions experienced by each animal and/or the effects of subsequent maternal behaviour on later physiology and behaviour. The applicant has been working with avian species for over 20 years and therefore is well placed to ensure the welfare of the animals in her care throughout the project.

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

Birds will typically experience a manipulation of their pre- and post-natal conditions, which may again be repeated in adulthood to look at how matching conditions across time can influence health and wellbeing. In the pre-natal stages, we will manipulate these conditions via changing the contents of the egg (e.g. to mimic altered hormone levels) or play sounds that mimic changes to the external environment (e.g. increased competition upon hatching). In the post-natal stages, we may also alter hormone levels via direct administration of these substances or alter environmental conditions by changing the food available to the birds, the light regimes they are exposed to or again altering the acoustic soundscape that they hear (e.g. playback of predator calls). Most of the work will be carried out in captive birds; birds we catch in the wild will have a more limited array of manipulations applied to them so we can ensure we use the most refined, practical and appropriate environmental challenges. We may also manipulate environmental conditions

during a single life phase to look at independent effects. These manipulations will be set to mimic natural changes in environmental or physiological environments that birds of a certain species might experience, for example any stress hormone doses will be carefully scaled so they mimic changes seen in animals that are experiencing natural acute stressors, such as a predator presence. Birds will only experience up to three of these manipulations across their lifetime.

We will also measure the behavioural responses of individuals to our manipulations. A range of behaviours will be assessed at each stage of the bird's life cycle. Tests include responses to a novel object placed in the home cage, responses to placement in a novel cage, responses to altered social situations (e.g. single housing or introduction to new social group), assessment of cognitive abilities (e.g. song learning and production, spatial memory, associative learning, social information use), assessment of social networks and breeding behaviour (e.g. incubation effort, nest building, mate feeding). some of these tasks can be modified for use in free living animals to test the same behavioural traits, e.g. the novel object test. This means we can compare across the two different protocols, but also test behaviour at the site of capture with no need to move the animal great distances.

We will then use some procedures to gather biological samples to look at health indicators. Firstly, blood samples will be taken to look at blood parameters, such as how well the immune system is functioning and also how well the body's other physiological systems are balanced. This can tell us how well an animal is coping in a certain environment and give us insight into the mechanisms that might underlie the ability to be resilient. To add to this investigation on mechanisms, we will also kill birds to take tissue from them, including the brain. In wild birds we will only do this in a small subsample of experiments and only when we have permission from other external agencies to do so. Here we can determine how the central regulatory systems have been affected and if early life conditions cause permanent impacts on the brain, or if compensation is possible.

We will follow birds in captivity for their whole lives, but in the wild we will track term as long as possible (sometimes using tracking devices). This means some animals will be in experiments that last up to 1 year or more, but they will not be exposed to our manipulations over that whole time. We will choose discrete periods to test their responses. Most experiments will last 4-16 weeks, with then some tracking of how the individuals develop and behave in later life.

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

All our manipulations are designed to mimic natural changes seen in the environment and as such they should only cause mild and transitory stress or discomfort. For example, blood sampling causes a short-term induction in pain when withdrawing blood, but birds recover fast and are able to return to their normal activities within minutes. Increasing or decreasing hormone concentrations can cause changes in growth rates or metabolism. However, we only use physiologically relevant doses that mimic natural changes and so these impacts are very unlikely to occur. We monitor birds very closely to make sure we are not causing distress and cease any procedures that might lead to this. At the end of all captive and some wild based experiments we will humanely euthanise animals to collect tissue.

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 our procedures are known to cause mild harms, with most having transitory impacts on the wellbeing of the animals. We estimate from past experience that 80% of birds will experience MILD harms, whilst the rest will be sub-threshold.

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?

In order to properly meet our experimental objectives experimental adjustment of developmental and adulthood conditions is necessary as is the long-term monitoring of many traits into adulthood.

Therefore, the need to follow living animals through time in this research is essential, since a major aim of the project is to understand how animals respond to environmental conditions and how these can influence a range of important traits throughout life. Past work on animals has shown that perturbations to developmental conditions can impact on the development of physiological systems, which can impact health and behaviour. This introduced the concept known as 'Developmental Programming'. However, most of this work has been undertaken in mammalian species in the laboratory focussed primarily on disease traits (Web of Science and PubMed searches show over 9000 publications in the last 5 years). This project focusses on bird species in both captivity and the wild, which are much less studied (searches indicate 750 publications in last 5 years). This is despite the fact they are uniquely able to answer fundamental questions around the role of developmental environments in altering health and wellbeing, due to their sealed egg that separates embryo from the mother after laying and allowing us to track the developing individual. Birds also allow us to target species that are used in laboratories (such as zebra finches), for food (such as chickens) and those used as indicators of environmental quality in the wild (such as seabirds). Birds give us the ability to feed directly back into the procedures and policies that keep them healthy and maintain good welfare. To be able to do this though we need to study the living animals in those environments.

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. Search terms vary depending on the experiment but can include: developmental programming; brain; bird; HPA axis; receptor density. In vitro methodologies are a possibility for looking at simple neural or organ responses to specific chemicals and computer aided technology, such as mathematical modelling could help to understand interactions between life stages and impacts at a population level. As this work aims to determine the impacts of environmental challenges on avian physiology, resilience and behaviour any alternative model would need to be able to provide a system that was directly relevant to this taxonomic group, living in comparably complex habitats, requiring comparable nutrition and possessing comparable physiology. In addition, the oviparous nature of birds, whilst making them a strong model for understanding developmental

programming also limits the potential use of alternative models that do not lay eggs. For this reason utilising humans as an alternative would not be appropriate. Other less sentient oviparous models, such as nematodes, may provide partial abilities to look at conserved physiological changes following altered developmental conditions, however these models do not provide the complexity of systems to understand the mechanisms that can drive developmental programming in free living animals and would not allow the indepth investigations on important welfare-linked behaviours in birds.

Why were they not suitable?

Possible alternatives when looking at the mechanisms include cell cultures or organoids. These are of limited value as we are interested in organism wide systems and networks, and we need to corroborate the impacts of the different environments on behaviour and wellbeing. We want to take a holistic approach to the whole organism. In addition, it is very difficult to properly simulate environmental change in these in vitro models, the manipulations currently available are very limited. They cannot capture the complexity of responses in a developing organism across multiple

physiological systems, but with the techniques we have developed we are able to determine the impact on multiple physiological systems in one organism. They also cannot capture the responses of a developing bird to external cues, such as noise. Finally computational models could be used to determine how different life stages react to environmental change, and potentially model population level effects. However individual based modes such as this still need the basic data on parameters such as changes to stress levels, range of responses etc in order to work effectively. Models can be very useful for generating hypotheses and where possible these are used. However current models are necessarily simplistic and so cannot simulate the complexity of the systems we work with well.

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. This includes collecting data on multiple behaviours, utilising blood samples to measure multiple factors and collecting multiple tissue types to facilitate sharing of tissue and multiple factor analyses. We choose the type and nature of experimental groups to enable us to answer specific questions, for example, where we will look at the impacts across pre-natal and post-natal life we will aim to create fully factorial designs that look at all possible interactions. We will always employ control groups raised in standard ways to compare the experimental groups to. We will utilise manipulations that mimic changes in the environments animals live and so each will be scaled according to the life stage and species being used.

We will then utilise statistical analyses to compare the control and experimental groups. Allocation to these different groups will be via random allocation, unless birds need to be

matched for age or size, in that case allocation will be random within these constraints. We will use blinding to treatment group as often as possible, animals will be given a specific ID number that does not denote their experimental group, except for in the main database that tracks procedures and treatments to allow later matching for statistical analyses. In some cases birds will need to be allocated an additional ID to facilitate allocation to a dosing regime or food manipulation when blinding is not possible. to ensure appropriate procedures are carried out (e.g. the correct dose is given, or food removed from correct cage). Some pilot studies may be necessary for trialling behavioural tasks, but these will be kept to a minimum as we already have a battery of validated tasks for the species involved in this research.

We use effect sizes for our experiments that have been reported by other researchers in an allied area or from our past data collection; these allow us to estimate how large an effect our manipulations may have on the animals and hence determine what sample size we need to detect a meaningful effect using the most appropriate number of animals. Where necessary we will take advanced statistical advice and will regularly review sample sizes and the effect sizes for other experiments. Where possible we will use a longitudinal approach, tracking animals progress over time, this is known to reduce the number of animals required to gain meaningful results.

Different techniques, behavioural tasks and species habitats produce different patterns of data so group sizes will vary to ensure meaningful results but on average most studies will use 10-20 animals per group, which we have estimated from past experience of running comparable experiments. We aim to run multifactorial experiments with the proposed species, often these can entail 4-5 groups in captive species (including controls), which suggests a usage of 100 animals per year. We aim to run poultry experiments each year, giving a total maximum over the 5 year project of 500 animals per species. For the zebra finch we aim to run three experiments over the 5 year period, totalling a maximum of 300 birds. Running experiments in the wild is more time consuming and requires a large amount of monitoring and tracking of individuals who may be spatially far apart. Based on past experience 50 birds per species per year is a feasible number to capture, manipulate and monitor and ensure animal wellbeing is prioritised. We will scale our experiments (reduced number of groups) or run multi-year experiments in order to create robust and feasible designs that answer our research questions in these species.

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

Where possible we use longitudinal studies, where we track animals over a specific time, this increases the power of the work and means we reduce the number of animals used. In addition, we have refined several techniques and protocols over the years. For example, use direct manipulation of egg hormones rather than manipulate the environment of the mother where possible, and this reduces the overall number of animals used in experiments.

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.

We will limit sources of variation in captive species, by maintaining environmental conditions such as temperature and humidity and standardising husbandry and enclosure dimensions, unless alteration is part of the experiment. In the wild there is naturally more variation across environmental variables that we cannot standardise. However we do collect data on changes in variable such as weather to allow

us to control statistically in our analyses for this variation. Where possible also we conduct an experiment within a single POLE, reducing some variation across the population, or we again add the POLE identity to our statistical models as a control. In all experiments we will utilise standard techniques to collect biological samples, aim to standardise the age, weight and size of birds at specific monitoring points (e.g. when recording behaviour).

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 where appropriate to test new methods and behavioural tests to ensure birds will engage with any new equipment, for example. These will also give us the data we need to make calculations of group sizes for future studies.

We endeavour to bring animals in at the egg/embryo stage from reputable suppliers with excellent welfare standards, to avoid keeping more adult birds than necessary in breeding environments for prolonged periods. Where we do breed animals, we will use efficient practices to optimise the number of animals bred.

We also make the most use of each animal as possible by freezing tissue at the end of experiments. This is a valuable resource for us and other researchers who can make use of the tissue to test new hypotheses about how environments can alter neural and peripheral 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.

We will utilise bird species for the proposed work. We will use experimental manipulation of environmental conditions (e.g. hormone manipulation, food reduction) coupled with natural experiments (e.g. urban vs rural habitats), followed by methods to detect the birds physiological, neural and behavioural responses to these challenges. In each of these cases the maximum severity of the technique is mild. We will scale all manipulations to mimic the natural world or the environments they encounter in captivity and also study birds in their natural habitats alongside captive species of socioeconomic importance. We use these 'natural' manipulations because we are interested in how the animals respond to the world they encounter. By using these carefully scaled manipulations we ensure we cause the least pain, suffering or lasting harm to the animals.

The project studies multiple bird species, this is so we can compare birds with different ecologies in the wild to get a full picture of which environmental conditions are most important in mediating health and success. In the wild we focus on seabird species, as these are indicators of environmental quality, nest in large numbers and their chicks are accessible so we can monitor their health more easily and ensure the least harm comes to them. The species we have chosen differ in their niche use, particularly in terms of foraging and diet, but often nest in similar areas. This allows us a powerful comparison across ecologies, potentially enabling us to draw wider conclusions about which

environmental variables might have the most influence on species across the World. We also work with agencies such as the British Trust for Ornithology, who are experts in tracking and monitoring bird species to ensure we utilise best practice in the field at all times. It is important to study these birds in their natural environment to make sure we gain a true understanding of the role of environmental conditions in mediating their health. Bringing birds into captivity to study them would significantly increase stress and potential harm.

In captivity we focus on two species types: the zebra finch, which is used widely in laboratories across the World due to it's vocal learning abilities that are similar to humans. Understanding how we can maximise their welfare is important given the numbers of birds used in research each year. We also plan to use poultry species, including the chicken, pheasant, Japanese quail and guinea fowl. These birds are all used in parts of the World for food, be that meat and/or eggs and thus are of significant socioeconomic importance. Whilst they are all poultry they also differ in some physiological and behavioural traits and in their husbandry systems. Gaining an understanding of how early life can impact each of these will allow us to understand species specific requirements, but also allow important common threads to be identified which could help other species. We do not carry out work on any Schedule 2 species.

The methods we use will employ state of the art downstream analyses enabling us to determine multiple traits in an individual or even a single blood sample. We ensure to follow best practice when taking biological samples to ensure we use the least painful and fastest methodology, for example we utilise an aseptic approach when blood sampling, never re-use needles and only remove the amount required for the tests we intend to run to minimise the impact on the animal.

We will therefore 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 practice for all procedures. We will also keep up to date with current methods for analysing the brain and blood samples and adopt new methods that will result in lower levels of pain and distress wherever possible.

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

We will work with embryos as part of this project, however in order to meet our objectives we may also need to track these individuals into their later life stages.

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

We will not perform any surgical techniques on animals and all experimental manipulations will be carefully scaled to mimic natural variations that animals would encounter in their environment. We will ensure to use the most refined methodologies for manipulations in the wild and in captivity. For example, the applicant has pioneered the use of direct hormone manipulations into fertile eggs rather than manipulation the environment of the mother, thus facilitating the ability to manipulate a single hormone at a time to investigate mechanistic effects, and reducing the number of animals undergoing the manipulation. Captive birds will be socially housed unless housing manipulations are in progress, they will be provided with enrichment and health and behaviour monitored frequently. We will ensure that all animals are monitored daily even if no procedures have been undertaken, but following any proecdure more frequent monitoring will be undertaken. We will also monitor weight regularly and utilise score sheets to monitor body condition.

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

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.

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.

44. Circadian regulation of tissue immunity against infection and 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

HBV, Cancer, SARS-CoV-2, Vaccine, Circadian

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 project aims to investigate how circadian rhythms influence the immunity against viral infection and cancer. We will explore how time-of-day and circadian pathways affect the body's immune response and how this understanding can be used to enhance immunotherapy and the effectiveness of vaccines for viral 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?

This project focuses on diseases that present significant global health challenges. Chronic hepatitis B and liver cancer continue to have high incidence and mortality rates, highlighting the shortcomings of current therapies, which require lifelong use, cause toxicity, and fail to offer a complete cure. Similarly, SARS-CoV-2 remains a pressing global

health concern, as existing vaccines are unable to fully prevent infection, particularly against emerging variants.

Understanding how circadian rhythms influence immune response to viral infection and cancer could lead to novel treatment strategies, including optimising vaccine timing and developing circadian-based therapies. This work will also enhance the understanding of liver and local immune tolerance in chronic hepatitis B (CHB) and SARS-CoV-2 infection, and liver cancer. These insights may be applicable to other infectious diseases and cancers, with the potential to improve diagnostics and inform the choice of therapeutic approaches, ultimately saving lives and improving public health outcomes.

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

We aim to gain new insights into how the circadian clock influences organ-specific immunity to Hepatitis B Virus (HBV) and SARS-CoV-2 infection, as well as liver cancer, with a focus on tissueresident immunity. Our research will elucidate how circadian cycles impact antiviral and antitumour immunity and the mechanisms by which infections affect the tissue clock, subsequently influencing immune function. By understanding the role of circadian pathways in the T-cell exhaustion process, we can inform novel immune reconstitution strategies and enhance vaccine efficacy, especially by combining therapeutic vaccines with circadian modulators, targeting tissue-resident T cells.

Our research will address the mechanisms of hepatic immune suppression, defining prognostic markers and identifying targetable molecules for immunotherapy. This will improve diagnosis and treatment for patients with CHB and potentially enhance outcomes for other hepatotropic infections and autoimmune liver diseases. By contrasting T cell regulation in the liver and lung, we will identify unique and generalisable mechanisms of immune regulation, contributing to therapeutic strategies across these fields.

We expect to provide new insights into the liver microenvironments in chronic diseases, shedding light on the role of systemic versus local immune responses in tissues such as the liver and lung. These findings could contribute to the development of novel vaccines or immunotherapies for chronic HBV, SARS-CoV-2 and other coronavirus.

This work will also provide broader public health insights, showing how circadian rhythms influence adaptive immunity against viral infections and tumour. This understanding could aid epidemic modelling and inform public health measures. Furthermore, our research might lead to the development of new circadian-based treatments for various viral infections, with our findings disseminated through publications and presentations to the scientific community.

Lastly, we aim to optimise the time-of-day administration of a new generation SARS-CoV-2 vaccine that targets conserved viral regions and assesses the benefits of mucosal delivery. Such a vaccine could also induce broadly cross-reactive, durable infection-blocking mucosal immunity against other respiratory pathogens beyond the coronavirus family.

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

In the short term, researchers and healthcare professionals specialising in viral immunology may benefit from the new insights into the role of circadian rhythms in the immune response to HBV infection and liver cancer. These insights could lead to immediate improvements in immune reconstitution strategies and vaccine optimisation, potentially enhancing treatment outcomes for patients. Additionally, the findings could inform public health measures aimed at controlling viral outbreaks, providing valuable information for epidemic modelling and disease surveillance.

In the long term, the broader scientific community, including researchers, policymakers, and healthcare providers, stands to benefit from the dissemination of findings through publications and presentations. These insights could contribute to the development of new circadian-based treatments not only for HBV but also for other viral infections, potentially leading to more effective therapeutic interventions and improved public health outcomes globally. Moreover, patients with HBV or SARS-CoV-2 may ultimately benefit from optimised therapeutic vaccine timing and the development of novel circadianbased treatments, which could offer more effective and personalised approaches to managing their condition. Overall, the outputs of this project have the potential to generate significant advancements in our understanding of fundamental immunology and circadian biology, with far-reaching implications for both research and clinical practice.

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

We will actively seek collaboration with other research groups and institutions working in related fields, fostering interdisciplinary partnerships to leverage complementary expertise and resources. This collaborative approach will enhance the robustness of our research findings and accelerate the translation of discoveries into tangible outcomes.

Dissemination of new knowledge will be a priority. We will ensure that our findings are disseminated widely through various channels, including peer-reviewed publications, conference presentations, and workshops.

We will engage with stakeholders such as healthcare professionals, policymakers, and patient advocacy groups to ensure that our research findings reach those who can benefit from them most directly.

We recognise the importance of transparency and openness in scientific research. As such, we will be committed to publishing both successful and unsuccessful approaches, contributing to the collective knowledge base and preventing duplication of efforts. By sharing our findings openly, we can facilitate further innovation and collaboration within the scientific community.

Overall, by embracing collaboration, actively disseminating new knowledge, and promoting transparency, we aim to maximise the impact of our work and contribute meaningfully to advancements in the field of viral immunology, cancer and circadian biology.

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.

Mice are extensively studied and commonly employed in biomedical research, providing access to a wide range of genetic and immunological tools that aid in the investigation of intricate biological processes. Since it is impractical to collect human tissues at various times throughout the day, in vivo models are essential tools in circadian research, and mice present an excellent model due to their ease of circadian entrainment by light and food.

Our selection of mice is influenced by their susceptibility to AdHBV or AAV-HBV constructs, which allows for the induction of either acute resolving or chronic HBV infection depending on the administered dose. This adaptability is fundamental to our research goals, enabling exploration of both acute and chronic phases of HBV infection and the underlying mechanisms driving distinct disease outcomes.

Murine liver cancer models that recapitulate some key features of human liver cancer have been developed and are established in our laboratory. Adult mice are also appropriate for testing immune responses to vaccines, and the possibility of working with genetically altered animals allows for a deeper understanding of key circadian regulators, or immune molecules and cells, or increases the translational relevance of the work, for instance by predicting T cell responses in patients using mice that express human MHC.

Regarding life stages, our focus will primarily be on adult mice for our experiments. Adult mice are physiologically mature and more closely mimic the immune responses and disease progression observed in adult humans. Additionally, adult mice demonstrate greater robustness and are less susceptible to developmental variations, ensuring more consistent outcomes across experiments.

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

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.

The circadian rhythm of animals will be entrained by a light/dark cycle or a time-restricted feeding schedule, which will induce minimal adverse effects. Most animals in this protocol will receive a hepatotropic vector able to establish a model of chronic HBV infection and later be treated with immunotherapeutic interventions (typically 2-3 doses). Blood samples will be collected to confirm the establishment of chronic infection. At the end of the experiment (generally 30-40 days after infection), mice will be humanely killed and organs will be harvested for downstream analysis.

For the coronavirus vaccine work, naive mice will be immunised with 1-3 doses of candidate prophylactic pan-coronavirus vaccines via the intramuscular or mucosal route. The use of intramuscular injection is to closely mimic the method used for vaccine administration in humans, ensuring more relevant results. 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 15% of animals, 60-90 days after the last dose), mice will be humanely killed and organs will be harvested for analysis.

Mice may undergo tumour cell implantation followed by circadian entrainment and treatment at different circadian time points (typically 2-3 doses). We estimate that 70% of animals used for our liver tumour research will 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 humanely killed and organs will be harvested for analysis.

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

The expected impacts and adverse effects on animals during our project will vary depending on specific protocols and procedures. In general, animals may experience mild discomfort or transient pain from procedures such as injections and blood sampling.

Genetically altered animals are not expected to show harmful phenotypes. However, some may have an altered immune system, making them more susceptible to infections. Therefore, they will be housed in a barrier environment to minimise health risks.

The models of HBV infection used do not induce clinical signs in mice, and the immune interventions tested have previously been shown to be safe and well-tolerated in both mice and humans. Injected viral vectors and immune interventions are expected to be non-toxic or have limited toxicity at the doses used. Some animals may develop raised alanine aminotransferase (ALT) levels following vector injections, but these are typically well-tolerated. Humane endpoints will be established based on adverse clinical signs, and animals will be humanely killed if necessary to prevent unnecessary suffering.

The growth of tumours (subcutaneous liver tumour, syngeneic metastatic tumour and xenograft orthotopic liver tumour) can cause distress or pain due to local effects. Mice will be monitored daily for signs of toxicity or behavioural changes, and their weight will be assessed 2-3 times a week following infection.

Overall, injections are not expected to cause more than transient pain or 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)?

The anticipated severity levels and the distribution of animals across different categories vary depending on the specific protocols and procedures outlined in the project.

Animals used in this study are not expected to have any harmful phenotype. Consequently, the severity is minimal, and the proportion of animals encountering adverse effects is projected to be negligible.

For procedures involving viral vector injections or other associated procedures might undergo mild to moderate adverse effects, such as transient discomfort, elevated ALT levels, or alterations in behaviour. However, the proportion of animals falling into this category is anticipated to be low, as steps will be taken to mitigate distress and monitor for any signs of adverse reactions.

In cases where animals undergo procedures such as malignant cell transplantation or receiving injections could experience mild to moderate adverse effects, encompassing discomfort, swelling, or changes in behaviour. Similarly, the proportion of animals in this group is expected to be low, with measures in position to minimize distress and monitor for signs of adverse reactions.

Overall, we expect 25% of the animals to experience mild severity and 75% 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?

Animals are essential for this project to explore the intricate interplay between circadian biology and the immune system, as well as to evaluate the in vivo effects of therapeutic interventions and assess their translational potential. Additionally, both the therapeutic HBV vaccine and the mucosa-targeted pan-coronavirus vaccine necessitate in vivo murine models to refine their time-of-day administration. In vitro studies using human or murine tissue will be utilised whenever feasible.

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

Our research, guided by patient studies, aims to minimise animal usage by incorporating human tissue samples whenever feasible. We have evaluated the use of computational (in silico) models alongside in vitro approaches to further reduce animal testing. Through collaborations, we have developed tools for studying immune responses using engineered human T and NK cells in vitro. We also utilise human hepatoma cells expressing the HBV entry receptor NTCP and a 3D spheroid model for anti-tumour response studies. Circadian modulators will first undergo evaluation using these in silico and in vitro systems, with only the most promising candidates advancing to in vivo testing.

Why were they not suitable?

Non-animal alternatives such as in vitro cell and ex vivo culture systems were deemed unsuitable for this project due to the intricate nature of immune responses and the absence of an in situ tissue microenvironment. Computational models also proved inadequate in capturing the dynamic interactions within the immune system and physiological responses to therapeutic interventions. Furthermore, no in vitro models allow for the selective depletion or replacement of immune subsets and pathways in a relevant disease context, hindering the accurate evaluation of therapeutic targets and the assurance of safe pre-clinical testing. Therefore, studying immune responses in vitro significantly constrains our understanding of the interplay among immune system components and the development 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?

We determined the number of animals required by reviewing data from our collaborators' and our group's previous work, as well as relevant literature, ensuring that our sample size is sufficient to achieve statistical significance and reproducibility. Experimental groups will typically consist of 4-6 mice, which we've found to be necessary for attaining reproducible results and statistical significance.

To reduce experimental bias, we will implement measures such as blinding and randomisation of test subjects. This will involve using mice of different genders, distributing experimental groups across multiple cages, minimising turnover of experimenters or handlers, and confirming these measures through rigorous statistical analysis.

We carefully considered the specific objectives of each experiment and the anticipated outcomes to ensure that our sample size estimation adequately addresses the research questions.

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

We use well-established techniques such as multiparameter flow cytometry to extract comprehensive information from each sample, thereby reducing the overall number of animals required. Longitudinal monitoring methods, including serum ALT and HBV serology, enable repeated measurements from the same animals, thereby minimising the need for additional subjects.

To address variability and ensure sufficient sample sizes, we employ power calculations to estimate the number of mice needed for each protocol. To minimise experimental bias, we implement test subject blinding and randomisation, utilise both genders, select animals of similar weight and age from the same source, distribute experimental groups across multiple cages, and minimise experimenter turnover. Data are confirmed using rigorous statistical analysis.

We structured experiments to use the same animals for multiple purposes whenever feasible.

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 dedicated to minimising animal usage in our research by implementing multifaceted strategies aimed at maximising the utility and welfare of each animal. Our approach involves repurposing animals not needed for in vivo studies as organ donors for cell cultures, thereby enhancing the value derived from each specimen. Techniques such as multiparameter flow cytometry, capable of assessing numerous immune markers, and non-invasive imaging will be utilised to extract maximum data from each animal without increasing their suffering.

By leveraging current technologies and methodologies, such as serum ALT and HBV serology for indirect measurements, we aim to decrease the total number of animals required by extending the usage of individual animals across multiple experiments and parameters. This approach substantially reduces our overall reliance on animal models. Additionally, we will stay abreast of the latest advancements in our field to continually refine our methods and explore alternatives to animal models, thereby reducing our dependence on them.

We are committed to fostering collaboration and promoting data sharing within the scientific community to prevent redundant experiments and optimise the collective usage of research animals. Through these concerted efforts, we strive to uphold the highest standards of animal welfare while advancing our scientific understanding.

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 research will primarily utilise murine models, employing innovative methods such as a viral vector to induce chronic HBV infection and explore immune responses. Due to limitations in using larger, outbred animals like woodchucks and the impracticality of using naturally HBV-infected chimpanzees, we have chosen mice. These offer well-defined immune markers by utilising a viral vector that specifically targets murine liver. This approach enables us to model both acute and persistent HBV infections, aiding in understanding different immune responses.

Additionally, we may use C57BL/6 mice genetically modified to express human immune system markers, allowing precise tracking of T cell responses and paralleling studies in human subjects. Our approach ensures minimal animal suffering; previous experiments have shown no severe distress in mice, and all procedures are conducted by experienced researchers to minimise discomfort.

All animals are housed in optimal conditions with environmental enrichment to ensure their welfare and reduce experimental variability. Humane endpoints are in place for any unexpected adverse effects. The growth of orthotopic tumours is more likely to cause pain or weight loss than subcutaneous tumours. Additionally, tumour cell injection into the liver requires a small surgical procedure. Therefore, we will prioritise testing therapeutic interventions using the subcutaneous model and only proceed with the most promising therapies in the orthotopic model.

We can monitor tumour growth through in vivo, non-invasive imaging, including the In Vivo Imaging System (IVIS), and will use this to avoid excessive tumour burden. All handling and procedures will be performed by experienced researchers to minimise distress. Animals will be kept in well-maintained housing, with environmental enrichment such as nesting material provided, reducing experimental variability caused by environmental stresses like infections.

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

Our research employs murine models, selected for their well-defined immune markers and the availability of transgenic strains that facilitate the tracking of specific immune responses crucial to HBV infection. Mice are particularly suitable due to their similar liver and lung physiology, structure, and immunology to humans, making adult mice the optimal life stage for our experiments.

In addition, initial in vitro studies will inform subsequent animal experiments, thereby not only reducing the number of animals required but also aiding in the identification of pathways and therapeutic interventions with higher potential for successful translation to human applications. This approach allows us to maximise the utility of animal models while minimising the number needed and increasing the likelihood of clinically relevant findings.

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

We are dedicated to ensuring that all personnel involved in animal handling and experimentation receive comprehensive training to perform procedures with precision and care, thereby minimising potential distress to the animals. Our team will closely monitor the animals throughout the experimental process, promptly addressing any signs of discomfort or distress. We employ techniques such as subject blinding and randomisation to minimise bias and stress-related responses.

Regular veterinary health checks are conducted to monitor well-being and intervene if health issues arise, and we adhere to strict protocols for humane endpoints, ensuring prompt and humane euthanasia for animals experiencing significant distress. With years of experience conducting similar experiments, we have optimised our procedures to minimise distress and pain, such as following recommendations for performing injections under inhaled anaesthesia.

We continually collaborate with our NACWO and NVS to refine our anaesthetic and analgesic protocols, ensuring the highest standards of animal welfare are maintained throughout our research.

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

We are committed to upholding the highest standards of animal welfare and research integrity by adhering to various guidelines and resources:

ARRIVE guidelines: We will follow the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines, which offer comprehensive recommendations for the design and reporting of animal research to enhance reproducibility and transparency.

NC3Rs guidelines: We will consult guidance from the National Centre for the Replacement,

Refinement & Reduction of Animals in Research (NC3Rs), an organisation dedicated to promoting the 3Rs principles. Their publications provide valuable insights into refining experimental procedures to minimise animal suffering and improve welfare. In addition, our work on cancer models adheres to the best practices outlined in the 'Guidelines for the Welfare and Use of Animals in Cancer Research' (Workman et al., British Journal of Cancer, 2010, 102, 1555–1577).

Institutional and regulatory guidelines: We will adhere to the guidelines and regulations set forth by our institutional animal care and use committee (IACUC) or equivalent regulatory bodies. These guidelines ensure compliance with ethical standards and promote the responsible and humane use of animals in research. We will seek advice from our NACWO and NVS on best practice guidance for animal welfare and environmental enrichment, working closely with the animal facility staff to ensure recommendations are implemented and updated as needed. With support from the BSU staff, we will continue to carefully and routinely monitor our animals for any adverse effects.

Peer-reviewed literature: We will stay informed about relevant scientific literature and studies that showcase best practices in refining experimental procedures. By keeping abreast of advancements in animal welfare and refinement techniques, we can continuously improve our experimental protocols.

By integrating guidance from these sources, we aim to conduct experiments in the most refined and ethical manner possible, prioritising the welfare of the animals involved while ensuring the scientific integrity of our research.

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

We are dedicated to staying informed about advances in the 3Rs principles by consistently reviewing relevant literature, attending conferences and workshops, and engaging with field experts. Our efforts include staying updated through resources such as the NC3Rs newsletter and our institution's 3Rs newsfeed.

We will maintain close interactions with our Named Veterinary Surgeon (NVS), Named Animal Care and Welfare Officer (NACWO), and the staff at the Biological Services unit to enhance our knowledge of new advancements in animal welfare practices.

Furthermore, we are committed to integrating refined techniques and alternative methodologies into our practices to reduce reliance on animal models, thus minimising animal usage while upholding scientific rigour. Additionally, we will actively share relevant advancements with other groups in our department that perform animal work, fostering broader implementation of these important principles across our institution.

45. Splicing modulators as anticancer agents

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, alternative splicing

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?

Perform therapeutic proof-of-principle experiments to demonstrate that manipulation of a certain level of gene regulation named “alternative splicing” is a viable strategy for anti-cancer drugs 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?

In the last 20–30 years, there has been a revolution in anticancer treatments involving the rational design of targeted therapies against many molecular targets. While a complete cure of all cancers may never be achieved, the availability of a wide range of targeted therapies against multiple molecules, which may be used in both generalized and personalized therapeutics, will give rise to the possibility of transforming many cancers in chronic diseases, as they are managed more effectively.

There is therefore an important need to design and test new cancer drugs, especially with new mechanisms of action at molecular level. This project proposes to test anti-cancer drugs that work primarily manipulating a certain level of gene regulation named

“alternative splicing” that is hijacked by cancer cells. We hope that compounds that are able to modify this process will soon be part of the arsenal of anti-cancer treatments.

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

One of the main outputs will be generation of new information.

The drugs we are trying to develop relate to an important aspect of gene regulation, called “alternative splicing” - this is when different parts of a gene are included or excluded in the RNA (sister of DNA) - that codes for the protein. For example, if we take the sentence “Today is a good and bad day”, if we splice out some words it could read “Today is a good day”, or in another instance, “Today is a bad day”. Both are splicing variations of the same sentence (which denotes the gene), but each spliced sentence has a different meaning (which denotes the resulting proteins).

Alternative splicing (AS) has been described more than 50 years ago and it is quite well studied at biochemical level - in terms of how proteins and RNA interact to produce various forms of the same gene named "splice isoforms". However, only fairly recently, it has been realized that AS occurs in more than 94% of genes in humans and that splice isoforms may have very different functions similar to being encoded by different genes – this has cemented the role of AS as a very important level of gene regulation in the cell. Further-on it has been showed that during disease progression, including cancer, there are many isoforms that develop specifically, different from normal ones and therefore the idea of using manipulation of AS (reversal of isoforms to normal pattern) as a therapeutic avenue has arisen. This is a very novel field and this project proposal looks into adding further building blocks to our current knowledge.

We are focused on proof-of-concept experiments for development of a novel therapeutic class in cancer – the splicing modulators.

The research proposed will advance the field in several ways:

· define the role of some splicing regulators in cancer progression

· show importance of splicing regulators in vivo in tumours grown in laboratory animals (to mimic human tumours)

· proof-of-principle that AS and splicing regulators may be used to develop novel specific therapies for cancer

Another output will certainly be publications and presentations at scientific conferences. Our lab has a good track record of publishing in high-impact journals like Cancer Cell, Oncogene or Oncogenesis; I am invited every year to 9-10 international conferences to present my research in the cancer therapeutics area.

Finally, while it might not happen during the life of this PPL, the research described in this licence is in the cancer therapeutics area so there is a good chance that some of our target compounds will further be developed as drugs in future projects.

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

We envision the following beneficiaries:

Molecular and splicing biologists. Beside trying to develop novel therapeutics this project will elucidate how several compounds signal to the splicing machinery resulting in a group

of molecules that can be used as modulators of alternative splicing for further basic science studies. Many other molecules are active in tumour progression and undergo splicing and scientists studying these molecules and their splicing repertoire will also benefit.

Cancer biologists. Splicing control is under-investigated in many diseases and this proposal uses as a model-system tumours. Therefore the findings will be of great interest to cancer biologists who may link findings of this proposal related to splicing to tumour biology in the systems they are studying and therefore may result in new understanding of biology of cancer and/or novel therapeutic ideas in downstream studies

The Pharmaceutical industry may investigate the suitability of splicing control as a novel therapeutic strategy or use the link between splicing and cancer biology to test for potential effects of drugs.

Biochemists and chemists and those investigating how chemical properties affect the interaction of molecules within the complex that does the gene regulation step named “alternative splicing”.

Clinicians. One of the aim of the project is to develop anti-angiogenic drugs based on manipulation of splicing and therefore may be investigated further by clinicians working in the field

Who else might benefit from this research and how:

Impact on health and welfare: patients – this research aims to develop anti-cancer drugs but at the same time will further our understanding of basic molecular mechanism of cancer progression; therefore it has the potential to benefit to patients with various cancers within a time-frame of 10-15 years

Commercial Impact: the findings in this project may be taken together with industry partners towards development of new therapies in an area very much under-explored at the moment – modulation of alternative splicing; we are currently exploring together with our Innovation, Impact and Bussiness department on how to best take forward this work towards patent filing

Impact on services - NHS practitioners: there is potential for a whole novel class of therapeutics based on splicing that might help health practitioners in the future to expand the arsenal of tools to fight debilitating diseases such as cancer; if successful, these new drugs may be in the clinic in 10-15 years

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

The work described in this licence involves quite a few collaborations with other labs both from our and from other universities.

Findings from this work will be made available to other scientists through publication in peer-reviewed journals and presentations at scientific conferences. Under the previous licence we have published 10 papers and there are 4 others either submitted or in preparation. The licence holder has been invited to present these findings at >30 international conferences and members of his group presented the data at 6 more conferences.

Some of the work done under previous PPLs is continued by a biomedical company : https://www.exonate.com/technology/science/

The work has been featured in many media outlets and I have been invited to talk at the Bristol association of patients with prostate cancer (PROSPECT).

Species and numbers of animals expected to be used

- Mice: 1750

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.

Tumours are as complex in structure and organization as organs are. Though cancer cells form the bulk of the tumours volume they contain other types of cells, e.g. inflammatory cells as well as a sophisticated vasculature. An important component of the ability of tumours to grow is based on the interactions with the host organism and the structures surrounding them (tumour microenvironment). Therefore, it is essential to study tumour biology in vivo in animal models as only limited information may be obtained from culturing cancer cells in vitro. Additionally, metastasis is a process that happens in vivo.

Our lab is doing basic research in oncology and for this purpose the most use animal models are mice. We only use adult mice in our experiments.

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

In the most typical experiment done under this licence, mice are firstly injected subcutaneously in the flank with human tumour cells. After 7-10 days, depending on the tumour type, a lump appears under the skin in the flank region that was injected, which is the growing tumour. We measure this tumour growth twice weekly by using a calliper and when it reaches 3/3mm in diameters we assign the mice to either a control or an experimental group. These groups will be injected typically 3 times a week intraperitoneally with either inactive substance (control) or the drug we are studying (experimental). Tumour growth continues to be monitored to assess the effect of the treatment; typically the experiment is about a month long.

Sometimes the human cancer cells do not grow if injected subcutaneously in mice and they need to be injected in the organ were they came from - so called "orthotopic" models. In this case, under general anaesthesia and strict aseptic conditions, an incision is made either in the flank or the abdominal midline and cancer cells are injected in an organ (e.g prostate or under kidney capsule). In these cases the cells are usually tagged wiutha. marker that emits light and the mice can be anaesthetised and imaged periodically in a special chamber to monitor the tumour growth.

Sometimes transgenic models may be used as well. These are mice that have a certain genetic modification that results in the mouse developing a certain type of tumour. Administration of substances and tumour growth monitoring is done as described above.

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

NUDE MICE. Adverse effects: this mouse model is immunocompromised, such as human tumour cells may be implanted and are not rejected; theoretically there is the potential of higher risk of infections than in other mice strains.

GENERAL ANAESTHESIA. This may be used in all mice. It will be done only with inhalable anaesthetic (isoflurane); typical duration is 30 seconds – 1 minute for injections of tumour cells subcutaneously. If surgery is performed the duration will be longer – typical 15-30min, . Adverse effects: Delayed recovery from anaesthesia. Death under anaesthetic. Likelihood less than 1%.

INJECTION OF CELLS. Adverse effect: Infection. Likelihood: less than 0.1%

TUMOUR GROWTH. Adverse effect: Tumour growth under the skin may result in ulceration. Likelihood less than 1%.

MEASUREMENT OF TUMOUR GROWTH. Measurement of diameter using callipers is not expected to have any adverse effects.

ADMINISTRATION OF SUBSTANCES. Adverse effects: Toxicity. Likelihood less than 1%.

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 are used under this licence.

We expect most mice under procedures (90%) to be in the "mild" category; 10% (the ones that need surgery) will be in the "moderate" 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?

Tumours are complex structures with the architecture similar to an organ; while the bulk of the tumours are formed by a single cancerous cell type, they do have multiple other cells in composition (e.g.

stromal cells, inflammatory cells, vasculature). Growth and metastasis of tumours are dependent not only on the cancer cells per se but also on the interactions between cancer cells and the rest of the cells in the tumour microenvironment as well as the host organism. A considerable amount of cancerrelated research is done in cell culture in vitro. While this is very valuable it is also limited because of the complexity of tumours in vivo. In particular, with respect to splice factors that are the subject of research of this project, functions that are described in vitro many times prove to be redundant when studied in an in vivo context. Therefore, to fully understand tumour biology it is essential that experiments are performed in vivo.

There are studies on alternative splicing in simpler organisms, such as a fly model named Drosophila, that is frequently used in research. However, it works only for very basic biology questions, as the conservation in alternative splicing between humans and

Drosophila is very low. Additionally, we are interested in how to use alternative splicing to design anti-cancer drugs, which would be impossible to study in Drosophila.

There are cancer studies performed in zebrafish for example; however, this model is useful for more simplistic, straightforward studies than ours; additionally, alternative splicing is very little conserved in zebrafish compared to humans. Therefore, the simplest model we can use for these studies is the mouse.

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

We have used a large number of cancer cell lines (e.g. prostate, breast, colon). In fact we continue to use these always for our preliminary studies before using animals.

Why were they not suitable?

Tumours are complex structures with the architecture similar to an organ; while the bulk of the tumours are formed by a single cancerous cell type, they do have multiple other cells in composition (e.g. stromal cells, inflammatory cells, vasculature). Growth and metastasis of tumours are dependent not only on the cancer cells per se but also on the interactions between cancer cells and the rest of the cells in the tumour microenvironment as well as the host organism. A considerable amount of cancerrelated research is done in cell culture in vitro. While this is very valuable it is also limited because of the complexity of tumours in vivo. In particular, with respect to splice factors that are the subject of research of this project, functions that are described in vitro many times prove to be redundant when studied in an in vivo context. Therefore, to fully understand tumour biology it is essential that experiments are performed in vivo.

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 worked with mice xenografts and transgenic models for over 15 years; as such, I have a wealth of previous data to be able to estimate using statistical models how many mice we need for a certain experiment. I have also used my previous HO returns to estimate number of mice needed.

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

Experiments will be done using paired analysis or repetitive analysis. The latter has greater statistical power, and animals need only be killed at the end of experiments rather than at each time point drastically reducing the numbers of animals used – e.g. from 72 (for an experiment where 6 mice are culled, tumours harvested and measured on each day for 12 days), to 6 (6 mice with tumours imaged and measured by calliper every day for 12 days). Power analysis is used to minimize animal numbers. Block design is used to correct for multiple treatments, and statistical power analysis carried out in conjunction with local statistical experts. Scientific rigor makes it compulsory to use vehicles in which substances are dissolved for the therapeutic-type of experiments; however these will be kept at minimum especially if the vehicle is saline.

In a typical experiment in which we want to enquire whether a certain chemical decreases tumour growth, we would use a control group which will be injected with saline and an experimental group treated with the chemical at the concentration established in previous pilot experiments. To minimize mice numbers, we will group at least 3 chemicals together for the same control group, meaning working with 4 groups of 8 mice at a time. Knowing the estimated number of mice in a group and how many conditions we will test (chemicals or cell types with genes manipulations) we have estimated the numbers of mice we believe we need over the duration of the project and for each protocol (section E).

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

Transgenic mice numbers will be kept to a minimum by using crossing designs that result in minimal animals (e.g. homozygous crosses where possible), demand will be assessed before breeding and crossing, colonies will only be maintained while there is an experimental plan and funding allocated, breeding schedules will be informed by the fecundity and productivity statistics calculated from previous experience of breeding any of the models to be used.

One particular strength of the project is the use of in vivo imaging using a Xenogen device. In some of our studies we need to implant tumour cells in internal organs like prostate and kidney; it is therefore quite hard to measure their growth without special devices. The Xenogen device allows for repeated imaging at various time-points during the development of metastasis; from these repeated

measurements, metastasis foci growth curves are compiled and compared; because we are comparing these curves in real time, we will need fewer mice than the classical method used without imaging, in which mice are simply culled at the end, when humane points are reached, organs dissected, and metastatic foci counted.

All mice in which tumours do not develop according to expected time-frames will be culled.

Since subcutaneous injection of tumour cells will be done most of the times under anaesthesia, this will result in tumour placement and growth to be more accurate, therefore more results can be gained from each animal. This will result in reduction of total animals needed.

Pilot studies are always designed when pursuing a novel direction of 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.

Two animal models will be used:

Nude mice – mice that have been genetically altered to inhibit their immune system; these mice are widely used and considered the best model across the world for human cancer

cells implantation studies; the main reason is that human cancer cells would not be able to grow in a different species if the immune system would be intact; in particular for my project, this is the best model possible – we are investigating the response of various types of human cancers to different chemicals; we can grow human tumours only in this model

Genetically altered mice that harbour a so-called “splicing reporter” – a tool through which we can understand gene regulation in various tissues and cells just by imaging– compared to other species mice are considered the easiest to be manipulated genetically and therefore we used mice to genetically insert these reporters; similarly for another transgenic model for neurofibromatosis (a neural tumour)

We are using mouse tumour protocols that have previously been used to study growth inhibition to reduce the number of experiments. Thus mice will be killed before the tumour load becomes large enough to impair health in these animals, thereby reducing the likelihood of pain, suffering, distress or harm. The following models are used:

subcutaneous implantation – tumour cells are implanted under the skin; this is the simplest model we can use to grow human tumours – there is limited suffering for the mice and they are killed when

tumours are fairly small in size; we will use most of the time general anaesthesia, so the placement and shape of the tumours are more accurate and the chances to grow multiple tumours in an animal is reduced, therefore a better experience for the animal overall

orthotopic models for prostate, breast and kidney – sometimes tumours do not grow unless reimplanted back in the organ they originated from; this is done with surgery under anaesthesia and aseptic conditions; the project leader has more than 15 years experience with these models and the procedures and monitoring are designed so there is limited suffering for the mice

The substances that we want to work with in this project are either known drugs or are chemicals that have been developed with the intention to become drugs – they are therefore known to not be in general toxic. They have been tested extensively in cell culture and have been shown that are able to inhibit various functions of cancer cells. They are therefore strong candidates to be developed in the future as anti-cancer drugs. We hope to show that they inhibit tumour growth or spread of cancer cells in the organism.

Because these substances are known and studies before, we do not expect any important unwanted effects when administered in mice. However, for added safety, for any substance that we did not use before in mice, we will follow a staged approach, in which we will perform pilot studies for tolerability.

The introduction of repetitive imaging procedures reduces the number of animals that need to be used for tumour experiments and reduces the burden on those animals. Tumours can be detected when smaller than palpable, and metastases can be imaged before signs of distress occur. Since the objectives of these experiments are to determine the mechanisms underlying splice factor importance for tumour growth and metastasis in animal models of disease we will be investigating the early time points of tumour growth, when the least adverse effects are seen. Therefore these experiments are designed to cause the least pain, suffering, distress or lasting harm possible to achieve the objective. Furthermore, if the animals appear to be suffering, in pain or the tumours show evidence of harming the animal, the experiment will be terminated by killing the animal.

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

Non-mammalian organisms like zebrafish have been used in tumour xenograft research; however, they are appropriate only for very basic and incipient studies as tumours do not form the complexity (i.e.

similar to organs) that is seen in mammalian organisms; the tumour microenvironment (including blood vessels and stroma cells) is considered crucial for how a tumour develops and responds to treatment. Tumour xenografts and treatments need to be followed usually for an average of 4-6 weeks, which makes implantation in embryos inadequate.

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

A score sheet will be implemented upon administration of substances and adverse effects monitored daily either by the academic licensee or by the licensed animal unit staff. All animals will be monitored by a licensee at least once per day (at least twice per day for new substances) for signs of ill health or distress that might be caused by these substances, such as shivering, listlessness, alteration of normal grooming, eating or drinking. If such signs appear, the advice of the NACWO and/or NVS will be sought, the animal will be weighed, and a record of the weights will be kept on the cage label. The initial concentration of substances used in mice will be at the low-end range, will be done in pilot experiments and then increased progressively if no therapeutic effect is seen. The total volume of distribution of the substance in the mouse is calculated based on knowing that a mouse of 25 grams has on average 2 millilitres of blood and therefore we can estimate the amount of substance we inject in the mouse to correspond to the concentrations determined in the in vitro experiments. If we do not see therapeutic effects at the low-starting doses, the following increases in dosage will be done under the principles of single-ascending dose and multiple-ascending doses used in phase I clinical trials. Moreover, the substances we are using are constantly modified and refined by our close collaborations with chemical biologists to be able to obtain formulations that are more potent at lower concentrations and therefore try to minimize side-effects. Animals will be followed closely by a licensee, at least once daily. At any point, if the animals appear to be in pain or the tumours show evidence of harming the animal, the experiment will be terminated by killing the animal.

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

- OBSERVE: guidelines for the refinement of rodent cancer models; published in Nature Protocols, July 2024 https://www.nature.com/articles/s41596-024-00998-w

- Guidelines for the welfare and use of animals in cancer research, pubklished in British Journal of Cancer, 2010 https://pubmed.ncbi.nlm.nih.gov/20502460/

- Guiding Principles for Preparing for and Undertaking Aseptic Surgery https://lasa.co.uk/wp-content/uploads/2018/05/Aseptic-Surgery.pdf

- PREPARE: guidelines for planning animal research and testing https://pubmed.ncbi.nlm.nih.gov/28771074/

- The ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) https://arriveguidelines.org/

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

I regularly check the NC3 website and follow their newsletter; we are also updated periodically by our establishment licence holder about news in the 3R area as well as available symposia.

46. Exploring the Physiological Role of RNA Interference

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

immunity, viruses, RNA, stem cells, 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?

The aim of this project is to characterise and harness a defence mechanism called antiviral RNA interference in vivo, which has been recently uncovered to be active in mammals.

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?

Viruses constitute a constant threat to the human population as illustrated by the recent pandemic outbreaks caused by SARS-Cov2. To develop innovative therapeutic approaches to combat these infections, it is essential to understand the full repertoire of defences available in our cells. There is huge scope for discovery in the field of RNA

interference since this defence system has only recently been shown to be antiviral in mammals. Knowledge generated through this project will improve our understanding of our immune system and our ability to boost natural antiviral mechanisms through novel strategies for antiviral therapeutics and vaccine designs.

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

This project will provide essential knowledge about how the recently uncovered RNA interference pathway contributes to antiviral immunity in mammals and how it can be utilised for antiviral therapies. Findings of this project will be published in peer-reviewed scientific articles and innovative discoveries will be protected with an intellectual properties for their potential use in therapeutic strategies such as the improvement of vaccine efficiency.

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

This research programme will have a broad impact across a range of disciplines and contribute to the understanding of our immune system and precipitate innovative strategies for antiviral therapies. In the short term, this research will directly influence the academic community within the innate immunity field as it will provide wide-ranging insight into how this antiviral defence mechanism works, in which type of cells it is active and how it impacts viral infection either in newborns or adults. In the long term, the identification of a novel antiviral mechanism active in vivo and how it is counteracted by viral proteins will impact the pharmaceutical industry by providing novel targets for antiviral drugs. Results of this project will also benefit the industry sector through potential novel strategies to increase RNA-based vaccines' efficiency.

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

To disseminate this work to the wider research community, our findings will be presented at both national and international conferences. We will also report our research findings as preprints on dedicated online archive service such as bioRxyv or PeerJ Preprints to share rapidly our results and then submit in peer-reviewed and Open access journals. We will continue to share conceptual advances and shifts in research approaches by contributing review articles at timely intervals. The aim of this project addresses a longstanding question in the field and therefore negative results will equally be important for the scientific community and will therefore be published as well. I will also communicate my findings via our instutional media resources to generate news and publicity across our community , managing an extensive publishing programme for internal communication through newsletters and also for communications to external audiences through publications that showcase research being carried out in our teams..

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.

While the antiviral activity of RNA interference (RNAi) has been detected in cell culture, the key question is to define how much RNAi helps to protect living animals from infections. I first detected activities of this antiviral mechanism in mouse embryonic stem cells and then later on various types on mouse and human cells grown in culture. It is now primordial to test whether our findings in vitro are also observed using a living organism to then develop novel strategies to harness this mechanism of protection. The use of mice is therefore necessary to have a model in which to address the in vivo functional importance of this antiviral defence mechanism. Previous studies show signs of activity of RNAi in newborn mice, yet it has not be shown whether this activation was impacting the ability of viruses to replicate. Interestingly, the role of RNAi in adulthood has not been addressed yet. We will therefore need to test the antiviral activity of RNAi in adulthood.

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

Mice will received or not an injection of a compound or a control reagent and then will be either infected with a virus or a preparation of mRNA-containing lipid nanoparticles (LNPs). Then the immune response as well as the virus accumulation will be assessed at a defined time-point 1 to 10 days post infections/injection.

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

Mice will be infected with mild viral pathogens. The selection of viral agents to be used is aimed at producing the mildest reactions with a transient manifestation and an expected self-recovery.

Mice will be monitored daily throughout the course of disease, weight loss and clinical parameters will be recorded, and some mice may reach the humane end points described below.

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 are moderate and we estimate that less than 20% of mice will reach this limit.

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?

While the antiviral activity of RNA interference (RNAi) has been detected in cell culture, the key question is to define how much RNAi helps to protect living animals from infections with viruses or how much it impacts on technologies based on self-amplifying RNAs. I first detected activities of this antiviral mechanism in mouse embryonic stem cells and then later on various types on mouse and human cells grown in culture. It is now primordial to test whether our findings in vitro are also observed using a living organism to then develop novel strategies to harness this mechanism of protection. The use of mice is therefore necessary to have a model in which to address the in vivo functional importance of this antiviral defence mechanism.

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

In my first postdoctoral studies I studied antiviral RNAi in mouse embryonic stem cells and monitored its activity upon differentiation. I then analysed this pathway in various human and mouse cell lines during my second postdoctoral studies and then in my lab.

Why were they not suitable?

Numerous research have been done over the last two decades using various cell lines, primary cells and stem cells. The activity of RNAi is variable depending on the cell types. It is now key to understand its functional importance in vivo and study in which tissue and cells RNAi is active and how it impacts various physiological processes such as the resistance to viruses.

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 evaluated based on requirements for breeding and experiments. To assess the antiviral role of RNAi, we need to generate control (RNAi-competent mice) and experimental groups (RNA-deficient mice). This will involve 3 strains, which after 3 crossings to generate the two mouse strains which upon a single crossing (2 breeding trios) generate a progeny composed of mice from the control group or mice from the experimental group. I evaluated to use approximately 1000 animals for the maintenance and breeding of the necessary strains for the duration of the project.

These two groups (n=6/group) will be treated with compounds that are use to modulate some gene expression and phenotypes in the animals and then these animals will be challenged with viruses. I determined the size of each group by using the resource equation method to minimise the number of animals used while giving consistent results. For each infection, repeating experiments 3 times should result in 30-40 data points which should therefore be sufficiently powerful for statistics to be informative. Taking into accounts the maintenance, breeding, pilot experiments as well as the different viruses we will test (i.e. 2 viruses), I evaluate to use approximatively 250 animals.

For testing the impact of RNAi on the efficiency of replicon/self-amplifying RNAs to induce an immune response and protect from viral infections, we will need wild-type and/or mice defective in specific innate immune responses (i.e. type I Interferon pathway) and/or mice defective in RNAi in which we will inject self-amplyfing mRNAs/replicons. Per experiment, we will have 2 to 4 SAMs to test with 5 mice per group. To fully dissect the impact RNAi on SAMs, I evaluated to use 250 animals.

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

RNA interference (RNAi) is biological process that regulate the activity of genes by leading to targeted degradation of mRNA and that can moonlight as an antiviral pathway in mammalian cells. To test the contribution of RNAi in antiviral defence and on the efficiency of self-amplifying mRNAs (SAMs) that are mRNAs molecules that copy itself many times once inside the cells, we designed a crossing strategy such that we have breeding pairs from which all the progeny will be used in our experiments. The progeny will indeed have a genotype that will fall in either the control group or the experimental group. This will ensure that all the mice are used per experiment (males and females), which will minimise the number of mice used to obtain conclusive and significant results. Self-amplifying mRNAs (SAMs) will also be administrated to groups of wild-type mice and/or mice knockout for components of the type I interferon pathway. In these case, groups of mice (n=5/6) comprising males and females will be used, avoiding sex bias studies.

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

As mentioned above, an efficient breeding strategy will be used with breeding pairs providing progeny in which all mice can be used for our experiments. Pilot studies on a small group of animals will be performed to determine the optimal dose of substance (e.g.gene activators or virus required before carrying out larger experiments). In all experiments, we will harvest and store various tissues to always have the possibility to extract RNA, DNA and proteins and perform molecular analysis without the 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.

The proposed studies will use genetically modified animals to study the functional importance RNA interference as an antiviral system in mammals. An inducible genetic alteration was chosen to limit harm to the animals during development. This inducible system will allow to inactivate the pathway only prior an experimental procedure and will be left functional during breeding and maintenance. The mice will be supplemented with

food on the bottom of the cage prior to the injection with substance (e.g. gene activators) to prevent weight loss in the animals.

For the virus infections, we chose strains that are avirulent in mice therefore displaying limited symptoms and recovering naturally from the infections to reduce any unnecessary harm to the animals.

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

The selection of the animal species is justified by previous research activities in the area of inflammation and virology, where mouse models have been key to study and characterise responses to viral infections in mammals. The aim of the study is to test the contribution of the RNA interference pathway to immune responses towards viruses. The use of mouse model is essential for the available genetically modified animals required for our study and to study animals with a fully developed immune system.

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

Prior to the injection of the mice with gene activators, mice will be presented easy-access food on the bottom of the cages, to prevent weight loss by the substance. In any experiment mice will be handled at least a week prior to the start of the experiments, so that the mice are familiar to the researcher and stress during the experiment can be limited. During the experiment, the weight of the mice will be tracked as well as their behaviour. All cages will be supplemented with additional refinement (hanging tubes) to minimize stress and encourage normal behaviour. During infection studies mice will be followed up well established sepsis and infection scoring systems. These were adapted to a maximum moderate suffering of the animals. In addition, unnecessary pain and suffering during the infection studies will be prevented by the use of analgesia.

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

There are multiple guidelines present, as mentioned we will use the ARRIVE guidelines. Additionally, the experimental set-up will be matched to the PREPARE guidelines.

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

The host institution provides timely seminars focusing on animal welfare and refinement techniques, which will be followed by the PLL and PIL holders to ensure new advances on the 3Rs can be implemented in the ongoing project, In addition, the project will be evaluated internally every 6 months to keep up to date with guidelines such as PREPARE, ARRIVE 2.0 and other advances posted on

NC3Rs website to ensure the experimental lay out is in line with the most recent recommendations.

47. Bone Repair

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

Bone repair, Regeneration, Orthopaedics, Medical devices

Animal types	Life stages
Sheep	Juvenile, Adult
Rabbits	Juvenile, Adult
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 primary purpose of this service licence is to enable orthopaedic medical device companies to assess the safety and effectiveness of novel products, therapeutics and surgical techniques which are aimed at improving fracture repair and replacing bone that is lost due to either disease, trauma or surgery.

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?

Bone defects are serious conditions in which a part of a bone is damaged or missing owing to either disease, trauma or surgery, and needs to be repaired through interventional techniques and products such as bone grafting (using transplanted bone to repair and rebuild diseased or damaged bones), synthetic bone void fillers (synthetic materials designed to mimic bone which remove the need to transplant real bone) and/or bone adhesives/cements. In 2021 an estimated 2.2 million orthopaedic procedures involving bone grafting took place worldwide, with the incidence rate projected to increase by 13% each year.

Orthopaedic medical device companies continue to develop new and refined bone repair products intended to facilitate a superior bone repair, last longer than current products and/or lead to faster recovery times. All of these aspects will further improve patient's lives, reduce the need for early revision surgeries or further surgical interventions and thus reduce healthcare costs.

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

The repair therapies being evaluated within this service licence will be being developed with the intention of improving fracture repair and the replacement of bone that has been lost due to either disease, trauma or surgery.

It is expected that the data from successful studies (those showing no adverse effects as a result of the novel therapies and/or those showing improved bone repair) will be submitted to the relevant regulatory authorities for approval and launch of these new products.

The data from unsuccessful studies can be used to either stop the progression of work on technologies that do not appear provide the anticipated patient benefits or can be used to refine these technologies during their development.

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

This is a service licence which will enable orthopaedic medical device companies to access expertise and models that have been developed and validated over the last two decades in order to evaluate new products.

It is anticipated that members of the human population that have lost bone due to either disease, trauma or surgery will benefit. New repair therapies are likely to promote faster patient rehabilitation and better healing than current therapies and the resultant repair is expected to last longer. Longer lasting therapies will reduce the need for revision surgeries.

The surgical implantation of new repair therapies could be simpler as well as being more robust which will benefit surgeons.

Both of these benefits will in turn reduce the cost burden on healthcare providers.

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

The offering of validated bone repair models as a service to others means that numerous medical device companies will be able to evaluate their products in these models. Where confidentiality is not breached data will be shared across organisations and where possible, publications of the work conducted under this licence will be considered.

Species and numbers of animals expected to be used

Mice: 100

Rats: 100

Rabbits: 210

Sheep: 510

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 models contained within this service licence have long histories of use and are well documented in scientific literature and international testing standards for their intended purposes. The use of adult animals ensures that normal healing rates are sufficiently similar to the clinical situation.

The rodent model will be used to screen different materials prior to their assessment in larger animals. This model has been shown to be highly reproducible permitting the precise comparison of a variety of bone graft substitute materials within a non-load bearing site which enables accurate analysis of bone repair.

Clinical implants i.e. those designed for use in humans, can be evaluated in the larger animals without the need to scale up or down making the results much more likely to be accepted by a Regulatory Authority. In addition, the bones are of a size suitable for mechanical testing and histological analysis which are the endpoint measures that will be used to determine success.

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

Animals will be acclimatised to the facility and handling procedures prior to use.

Where sheep are being used they may have previously been pre-screened for use on another project and found to be unsuitable for that project. The pre-screening may have required imaging such as xrays under anaesthetic and the animals will only be used once they have fully recovered from the anaesthetic.

Blood may be taken according to general principles on blood sampling.

On the day of surgery animals will receive a pre-medication containing an analgesic (painkiller) and will then be anaesthetised for the surgical procedure.

The surgical procedure will be performed aseptically (in a sterile manner that is free from harmful bacteria and microorganisms) and will involve the implantation of bone grafts, bone void fillers and/or bone adhesives into bones to repair or fix surgically created defects. These defects could be in the hind limb, fore limb or skull. The harvesting of bone marrow from the rabbit or sheep pelvis or long bone could also be conducted so that the bone marrow can be incorporated into the repair therapy to improve its effectiveness. Surgical sites will be closed and the animals will be recovered from the anaesthetic. Post surgery painkillers will be used.

Following recovery, images such as x-rays may be taken to assess healing. An additional anaesthetic will be required each time images are taken, so because of this they will be taken no less than 1 week apart.

At the end of the procedure the animals will be humanely killed and the implant/host tissue construct will be removed for testing and analysis.

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

It is expected that there will be a degree of post-operative discomfort which will be controlled by painkillers. This isn't expected to last longer than 24-72 hrs following surgery.

Rodents and rabbits are expected to be group housed both pre and post-operatively but sheep will be single housed during the immediate post-operative period. This is to prevent injury before the sheep have fully recovered but as they are a herding animal this could cause some distress. To minimise this distress a line of sight will be provided to adjacent pen mates and group housing will normally be reintroduced 24-72 hrs following surgery. Re-introduction to group housing is expected to be without incident.

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 are expected to experience a moderate severity procedure.

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?

Bone repair is a complex process involving cellular repair mechanisms and inflammation. In-vitro (lab based) cell culture studies cannot replicate the in-vivo (in a living body) loading, physiological and anatomical conditions required to demonstrate the safety and efficacy of novel bone repair therapies, therefore animal studies are necessary in their development.

In addition, the endpoint measures required to study the strength of repair and the tissue/cell types making up that repair are biomechanical and histological, both requiring the use of living tissue of a size and structure appropriate to the intended clinical environment.

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

In-vitro cell culture studies involving the use of synthetic bone scaffolds either unloaded or under some load to try to emulate the clinical environment in which the final products will be used.

Why were they not suitable?

In-vitro cell culture studies are useful as a screening method to assess the effects of novel materials on the viability of cells. These types of studies will be used to screen out any potentially harmful structures or materials before healing/repair is studied.

However, in-vitro cell culture studies cannot replicate the in-vivo loading, physiological and anatomical conditions required to demonstrate the safety and efficacy of novel repair therapies, therefore animal studies are necessary in their development.

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 total number of animals has been estimated based on typical study sizes and the expected numbers of studies required for the 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?

As this is a service licence the experimental design phase for each required study has not yet happened.

When it does FRAME and NC3Rs guidance will be followed regarding reduction opportunities and the NC3Rs Experimental Design Tool (EDT) will be used where appropriate to inform the design of studies. Statisticians will be consulted in the planning stages of in-vivo studies to determine the appropriate study design, number of groups and number of animals required. Studies will typically be designed to 80% power, although this could differ, and could be designed, for example, as either superiority or noninferiority studies with appropriate limits depending on specific study objectives.

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

Historical data will be used to power studies where it exists. Otherwise pilot studies will be utilised to inform the design of subsequent pivotal studies.

Control items will be used as appropriate so that the results from novel test items can be compared against known controls. Test and control groups will be randomised within timepoints on each study and blinding methods will be used for both surgical implantation and for analysis where possible as this reduces potential bias towards test groups.

Animal variability will be reduced as much as possible by the sourcing of a consistent and reproducible supply and by incorporating acceptance criteria and weight ranges into study designs.

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 models contained within this service licence have long histories of use and are well documented in scientific literature and international testing standards for their intended purposes. For those reasons they are expected to cause the least pain, suffering, distress, or lasting harm to the animals.

The proposed models are as follows;

Rabbit segmental defect model - large segmental bone defects (where segments of long bones are missing, diseased or damaged) are a very serious problem in clinical practice. The primary purpose of this model is to determine the effectiveness of novel devices on bone regeneration. The model utilises a critical sized defect (one that would not spontaneously heal), which allows the effects of the test articles to be determined. The rabbit segmental defect model is described in American Society for Testing and Materials (ASTM) International standard F2721, Standard Guide for Pre-clinical in vivo Evaluation in Critical Size Segmental Bone Defects.

Rodent calvarial defect model – This is a well-established model for studying bone regeneration in a controlled environment and is one of the most commonly used experimental models for assessment of bone healing. It permits the assessment of bone formation in a critical sized defect in a non-loadbearing site (the flat and compact bones of the skullcap) in a reliable manner with minimal injury to the animals and provides preliminary screening data prior to further large animal and clinical trial assessment.

Sheep shallow defect model - The purpose of this model is to evaluate the efficacy of bone adhesives/cements under minimal to low weight bearing conditions. The bone piece is removed intact from a long bone in the hind limb and then replaced back into the defect held in place by either an adhesive or cement or a control item such as conventional orthopaedic hardware, e.g. screws.

Sheep contained defect model - This model is required for studying the biological response of host bone to a material in a large animal model. The sheep is a recognised orthopaedic model and the long bones are suitable anatomical sites for orthopaedic studies allowing implantation of similar sized implants to those used in humans. The model is described in International Standards Organisation (ISO) standard for Biological evaluation of medical devices 10993-6, Tests for local effects after implantation.

There is also a protocol to enable blood sampling from rabbits or sheep. The purpose of this protocol is to obtain blood for processing and compatibility experiments with prototype materials prior to any implantation studies being performed.

The animal models proposed have been developed, validated and refined under four previous successive Project Licences over a period of 20 years. Clinical implants i.e.

those designed for use in humans, can be evaluated in these models without the need to scale up or down making the results much more likely to be accepted by a Regulatory Authority.

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

The use of rodents ensures that a proportion of this work is conducted in a lower sentient species.

Use of adult rabbits and sheep ensures the models are clinically relevant for pivotal studies and, as both models are described in international standards, the data generated are more likely to be accepted by a Regulatory Authority.

Juveniles would have smaller limbs which may not have a sufficiently similar structure in which to study the required repair therapies. These would also heal much quicker potentially masking any improvements provided by the novel therapies. For these models less sentient species are unsuitable due to their size.

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

The animal models contained within this service licence have long histories of use and are well documented in scientific literature and international standards for their intended purposes. However, opportunities for further refinement will always be considered. Guidance from institutes such as NC3Rs will be followed where appropriate.

Acclimatisation periods will be utilised and refinements in post-operative care and pain management will be utilised where these are proven to reduce harms to the animals.

Animals will be group housed where possible and where single housing is required following a surgical procedure, a line of sight to a cage/pen mate will be provided by not having solid cage/pen sides. Group housing will be reintroduced as soon as possible after a surgical procedure which is expected to be without incident. Good ventilation is essential for large animals when housed indoors and when possible, these will be moved out to pasture.

Environmental enrichment methods will be utilised. In rodents and rabbits these can include the use nesting materials, materials to gnaw, shelters or tunnels, social interaction, scattering of treats for foraging, use of treat balls or puzzles, having something to climb and having the use of a mezzanine or loft space. In sheep these are mainly limited to providing a variety of feed and feeding methods. In addition to feeding good quality hay/haylage ad-lib a scoop of pelleted diet can be added for variety, mineral licks and additional feeds may also be provided and supplements e.g. beet or other appropriate fruit/veg may be fed as a form of environmental enrichment. The method of feeding can also be regularly changed to add variety.

Surgical implantations will be practiced and refined in cadaver tissues as required.

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

LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017), NC3Rs, ARRIVE and PREPARE guidelines will be followed where appropriate.

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

Through general literature review, review of NC3Rs website, dialogue with the Named Information Officer and Named Training and Competency Officer as well as other establishments.

48. Normal and abnormal brain plasticity

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

Brain plasticity, Neurodevelopmental disorders, Mental Health, Brain imaging, Behaviour

Animal types	Life stages
Rats	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult
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 how the brain changes from birth through to adulthood, both under normal development and in response to external influences and needs to adapt, as well as in cases of brain 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?

Disorders of the brain place a heavy burden on the individual and society, ranging from premature death to economic impact due to limited ability to find employment and including significant strain on healthcare, social services, and educational institutions. It is estimated that one in four people in the United Kingdom will experience a mental health

problem of some kind each year, and there are currently 14.7 million individuals living with a neurological disorder in the UK. These disorders include neurodevelopmental conditions like autism and attention deficit hyperactivity disorder (ADHD), mental health issues like depression and psychosis, and age related disorders like the dementias. These disorders share abnormalities or deficits in brain plasticity, or the ability of the brain to adapt and change in response to experience or new stimuli. Still, our understanding of these disorders is largely lacking, and treatment options are limited. Analysing neural anatomy, connectivity, and activity in these disorders and comparing them with that of a healthy brain will help uncover mechanisms that could lead to new or improved treatments.

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

Our major outputs from this project will be novel information on brain changes associated with psychiatric and neurological disorders such as autism and schizophrenia while also improving the techniques we use to measure the brain in both humans and animal models. Our work will be shared as publications in relevant scientific journals. We also plan to communicate our findings at regular conferences. Novel data will be made available for the research community for re-use as necessary. We expect that this work will ultimately lead to the development of novel treatments and, more immediately, the repurposing of existing treatments for novel uses. We further expect to create advances in brain imaging acquisition and analysis methods that will both improve the quality and type of data that can be acquired in rodents, result in refinements to rodent neuroscience, and translate to human brain imaging. Over the five years of this project, we expect to obtain novel information about the effects and mechanisms underlying 10 genes related to disorders of the brain while improving the fidelity of our brain imaging methods in at least three types of MRI acquisitions and testing the effects of at least two drugs. All our data will be released open access.

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

The main beneficiaries of this work will be the academic research community, in particular those working in understanding genomic variants in neuropsychiatric disorders, normal brain development and plasticity (the brain’s ability to change and adapt over time), and brain imaging technologies. We also expect that this research will have translatable benefits (such as the guidance of best practice and/or development of therapeutics). We envisage that our findings will be of interest to industry partners looking to develop and/or refine novel therapeutics for brain disorders.

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

The initial aim will be to share the data obtained through collaborations with expert scientists in the field of research, particularly in the field of rodent behaviours, as well as medicine and imaging physicists, and computer data scientists. Ultimately, we hope that much of the data will be made available for others to re-analyse and use. The data will be disseminated through preprint servers, publications, and presentations at appropriate forums. We will also share our work and consult with clinicians, in particular neurologists, psychiatrists, and paediatricians working in neurodevelopmental disorders and mental health, in order to maximize the translational value of our outcomes.

Furthermore, we will continue our past work of presenting and discussing our work with patient groups; we have found these discussions can further refine our experiments to have improved clinical relevance.

Negative results: we stand firmly behind the need to publish negative results, and to pre-register large pre-clinical studies. We aim to use platforms specifically designed to publish negative findings.

Species and numbers of animals expected to be used

- Mice: 11500
- Rats: 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 use mice and rats because ultimately behaviour arises as a consequence of co-ordinated activity within the brain, and the interaction with the internal and external environment. Therefore, non-animal alternatives are not appropriate and/or available. The inaccessibility of the brain in human participants makes the use of rodent models, where we can causally manipulate the genome and environment, essential. Most of our proposed work will be carried out in mice; rats will be used when their richer behavioural repertoire is particularly important, when there are advantages to studying their larger brain, or when genetic differences between rodents indicate the rat is a superior choice. For example, rats display a form of juvenile play not exhibited by mice, which can be an advantage when studying the brain circuitry underlying social behaviours relevant for autism. We will be studying brain and behaviour across newborn, juvenile and different adult stages, as different behaviours and disorders emerge at different times across the lifespan. For instance, social phenotypes (social phenotypes are characteristics that relate specifically to interactions with other animals) may be manifested from birth and/or change as the animals age.

We will use animals with and without genetic alterations as that allows us to assess the contributions of specific genes and pathways to neuropsychiatric or neurological diseases and fully characterize them in order to ascertain their usefulness as pre-clinical models of cognitive and behavioural decline.

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

Some of the animals we use will have their genes altered, for example, to be able to assess how their brain and learning behaviour is associated with neuropsychiatric or neurological diseases. In some cases these genetic alterations will need to be activated via a substance that allows genes to be activated either through their food or water, or an injection.

Some animals will have their blood sampled to better understand the effects of experimental interventions.

Mice and rats will undergo a combination of tests to understand the contribution of genomic variants to brain and behaviour. Each experiment will use a combination of tests over the life-span of the animals, most of which are non-invasive such as simple behavioural tests. Brain imaging, in particular MRI, will be the key method we use, and will usually involve general anaesthesia. Occasionally brain imaging will be done in combination with the injection of a contrast agent, and in some instances while intubated and under neuromuscular blockade on up to five occasions to facilitate neural readings.

Brain imaging and some behaviour testing will also be done in neonates and juveniles to monitor brain development.

Some animals will be exposed to behavioural tests that rely on aversive stimuli, such as a mild foot shock or the need to escape from water to find a platform in a maze.

Some animals will have restricted access to food or water in order to motivate them in some behavioural tests.

Some animals will be subjected to chronic mild stress to understand how stress impacts brain development and interacts with genes altered to model neuropsychiatric or neurological diseases.

Some animals will undergo brain surgery, to allow the insertion of wires for stimulation of the brain to assess brain function, or the insertion of a device that measures electric activity in the brain remotely.

Some animals will have their heads temporarily restrained while conscious, so that we can monitor their nerve cells and behaviour.

Some animals will be singly housed to monitor behaviour under very controlled conditions.

Some animals will undergo light manipulation or sleep deprivation to understand the impact of sleep or circadian rhythm disruption on brain and behaviour.

In order to explore underlying changes in brain chemistry some animals may also be given injections of neuroactive drugs or other kinds of substances.

In some cases drugs/substances will be administered via a surgically implanted device that allows the drugs to be released at a controlled, predetermined rate.

In some cases drugs/substances will be directly injected into the brain.

All animals will be killed by a humane method at the end of the experiments.

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

Genetic alterations in the mice and rats used in this project may lead to the development of

neurological or behavioural phenotypes that exist in the human conditions that are being modelled. It is expected that rodents modelling neuropsychiatric/neurological conditions may have delay and/or difficulty in developing skills relating to or involving the process of thinking and reasoning, communication and social interactions with other mice, and some sensorimotor functions, and these may have some adverse effects such as aggression, memory and sensory input such as the sense of smell, sight and hearing.

Imaging, behavioural tests, blood sampling, the insertion of small devices to deliver substances, administration of drugs or other substances, sleep deprivation, single housing, and light manipulation will only cause mild and transient discomfort.

Exposure to chronic stress (for example exposure to physical restraint or predator smells) may cause animals to move less and weight loss, which will be closely monitored.

Cranial surgeries, implantation of recording and stimulating electrodes, and intracranial substance administration can occasionally cause seizures. If seizures persist and animals do not respond to treatment, then the rodent 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)?

Mice

Subthreshold: 10% Mild: 10% Moderate: 80%

Rats

Subthreshold: 10% Mild: 10% Moderate: 80%

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 necessary to perform whole animal studies to achieve the experimental aims, since in all instances integrated physiological systems are affected. Behaviour is an emergent property of brain function, involving co-ordinated activity both within and external to the central nervous system. We thus need animal studies to study behaviour, brain circuits, and the relation between brain and behaviour.

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

We considered cell cultures and 3D brain organoids. We also considered relying on studies in human participants alone.

Why were they not suitable?

The whole organism is needed to examine how brain and behaviour interact and how brain and behavioural development change over the life course. The complex nature of how brain development interacts with processes such as the social environment, also means that non-mammalian species such as fruit flies or zebrafish do not model humans as well as rodents do. Rich information about basic physiology can indeed be obtained from flies and fish, so we will only engage in our proposed rodent studies where circuit mechanisms or brain-behaviour relations are important.

Human brain imaging (such as structural and functional MRI) studies will remain essential for our research programme, but do not allow for the precise mechanistic interrogation afforded by animal research. Given the rich correlational information that can be obtained

from human brain imaging studies we will only engage in animal studies where we can add mechanistic information difficult to obtain in human work alone.

Cell modelling, like using induced pluripotent stem cells, can help answer questions on how nerve cells work, but are only useful when we don't need to study behaviour or how entire brain circuits function. As with fly and fish models we will only engage in our proposed rodent studies where circuit mechanisms or brain-behaviour relations are important, as basic physiology can be addressed just as well or better in cell (or fly and fish) 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 number of animals outlined in this licence (11500 mice and 2000 rats) is based on our previous experimental experience from previous licenses.

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

Calculations based on previous data will be used for group sizes adequate for our experiments.

The combination of tests in each experiment will be designed to gather the most meaningful data to reduce the number of animals used. Similarly, we will perform different tests on the same individual to minimise differences between individual rodents that can influence the results and increase our ability to detect differences between treatments, thereby decreasing the number of animals used and increase the scientific utility of generated data.

The use of brain imaging and behavioural measurements over the life-span of the animal will allow us to get better data from a smaller number of rodents and to prove that the new/refined set of experimental plans is as accurate or better at detecting the same experimental outcome as the test it replaces over the progression of disease. Longitudinal data acquisition thus replaces the need for multiple separate cohorts at different timepoints while simultaneously improving statistical power.

We also adhere to the PRERPARE and ARRIVE guidelines and publish these alongside our results where necessary.

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 and cryopreservation will be used to minimise the number of rodents being produced for these studies. Genetically modified lines that have already been studied will be obtained from other sources to avoid remaking of lines, whenever possible.

Animals from the colony and tissue/samples will be shared with other researchers with overlapping interests, as well as the data produced by the project.

In addition, the data generated as part of this project, particularly brain imaging data, could be reinterrogated if new methodologies for analysis become available during the lifetime of this project.

Alternatively, as we build datasets that could be used as a baseline, these could be reused. For example, the use of computer modelling to predict patterns within big data is especially relevant to time dependent pattern of behaviours, where it is not necessary to generate new data for understanding what is 'the normal pattern' but compare new data generated in genetically modified models to historic data to tease out 'anomalies'.

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 examining brain and behavioural development across the lifespan in wild-type and genetically altered mouse and rat models. We will use rodents as experimental models since their physiology is well studied and they are genetically modifiable, plus, like people, their brain and behavioural development also depends on the interaction with the social environment (parenting, cagemates etc).

We will rely heavily on brain imaging, in particular MRI, to study brain anatomy, chemistry, connectivity, and function. These are the same techniques as would be used in humans, and allow for longitudinal monitoring of the animal across multiple time-scales.

Many of the behavioural methods used in this project are passive measures (home cage analysis) or measures of spontaneous natural behaviours in response to a stimulus where only a mild and transit discomfort is likely. Of those where training and learning are required, we use appetitively rewarded tasks where possible and mildly-aversive stimuli are necessary for valid outcomes. Rodent learning and problem solving at times needs proper motivation for optimal behaviour. This is generally accomplished via positive rewards such as condensed milk or similar treats and sometimes, if necessary by food or water restriction.

Certain methods of manipulating brain function, in particular techniques that use light to control cells in the brain, require the surgical implantation of stimulating devices. Similarly, to understand brain function, physiology, and structure we often have to manipulate it in the first place. This can include altering neurotransmitter function, blocking or enhancing neurotransmitter release, and similar techniques. Animals receive pain killers just as humans after surgeries.

Stress is an important consideration in disorders of the brain, as it can either trigger onset of symptoms or exacerbate existing symptoms. To understand this in animal models requires exposing the animals to a source of stress, usually consisting of unpredictable events or environments.

Given the important role of sleep disturbance in all psychiatric disorders as well as normal development, it is important to understand how sleep/wake states influence neural activity,

synaptic plasticity and behaviour. This at times requires light manipulation or sleep deprivation or studying animals with abnormal circadian function.

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

Brain and behavioural development involves complex co-ordinated interaction both within, and external (i.e. with mother and/or conspecifics) to the central nervous system, which are difficult to properly model in non-mammalian species such as flies or zebrafish, or anaesthetised animals. Finally, a major aim is to use MRI measures, which can be acquired in both rodents and humans, to translate our preclinical findings to humans; MRI cannot be obtained with adequate fidelity in less sentient species.

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 the animal has no additional stress, therefore animals are handled calmly, using tunnel handling (a technique where animals are guided through a small tube, rather than being picked up directly) or cupped hands where possible, and provided the time to get used to testing rooms and apparatuses alongside habituation to interacting with the experimenter ahead of time. Overall, we will continuously explore improved habituation protocols to minimize distress and will keenly follow advances in the 3Hs initiative (Housing, Handling, and Habituation - https://www.3hsinitiative.co.uk). Using brain imaging and passive monitoring through homecage analysis from birth to early adulthood and then coupling this with measures of learning later in life means we can examine behavioural development and adult learning in the same animal. This longitudinal approach will generate a statistically robust dataset and provide a better understanding of how gene variants associated with neuropsychiatric/neurological illness contribute to brain and behavioural changes and reduce the number or animals used.

Brain surgeries will be carried out under general anaesthesia. Animals will be given appropriate post- and peri- operative analgesia (pain relieving medication). Where possible this will be administered with food, for example nutella and food treated with sweet liquids, rather than by injection thus reducing the need for restraint and the possibility of multiple injections. The analgesic regime here aims to minimise pain, without having significant effects on outcome measures such as inflammation. All animals will be closely monitored to ensure there is no new development of pain as a result of their brain surgery.

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

We aim to conduct our experiments in accordance with the published NC3Rs guidelines, as well as Laboratory Animal Science Association (LASA, especially for asepsis and substance administration), ARRIVE and PREPARE guidelines.

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

Our establishment has regular NC3Rs update meetings throughout the year and regularly sends out information about national webinars and novel discoveries relating to NC3Rs research. We will also aim to keep ourselves updated by regularly visiting the NC3Rs and RSPCA websites. A key member of the group, the lead engineer/physicist, is a member of our establishment's "Animal Care and Ethical

Review: 3Rs subcommittee” and will therefore play a key role in ensuring implementation of 3Rs refinements.

In addition, members of the team at the establishment will attend specific conferences that focus on all aspects of the neurological and metabolic disease, from humans to model organisms, to in-vitro (experiments done outside the animal e.g. cell lines) and in-silico (computer modelling work such as artificial intelligence and computer learning). Any new developments which could impact these studies will be discussed with the teams at the establishment as well as experts in the field who may advise on this project.

49. Drug discovery in neurodegenerative diseases

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Neurodegeneration, Therapy, Drug discovery

Animal types	Life stages
Rats	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult
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 project is to outline the in vivo characteristics and efficacy of small molecule compounds generated within the drug discovery institute as potential therapeutics for neurodegenerative 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?

Neurodegeneration leading to dementia is a major unmet medical need and patient numbers are expected to increase with the aging population. Currently very few therapies can slow or reverse the disease process. This work will advance the discovery of new therapies, by providing support for evaluation of the uptake, metabolism and efficacy of novel potential drug candidates in rodents.

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

Our research will identify biological targets and develop potential therapeutics. by facilitating testing compounds/drugs that have been designed and pretested in lower model systems and show a beneficial therapeutic outcome and no harms. Our data will be generated in collaboration with key academic researchers and will be published, when possible, to inform the field. This will allow greater understanding of disease processes, enabling the identification of key targets and mechanisms which are effective and discarding those which are not viable to be modulated by drugs. This will ultimately enable better decision making by the pharmaceutical industry in deciding which targets to progress to development to then reach the clinics.

Outputs may include publications, dissemination via scientific meetings and congresses, pilot proof-ofconcept datasets for clinical translation of therapeutic candidates, and identification of novel compounds ready for further investigation and study.

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

The benefits to humans are the potential identification of new molecules which may become clinical drug candidates for the treatment of neurodegenerative diseases. This work will also advance science; testing of selective drug molecules targeting specific candidates thought to be involved in the disease process. This will allow researchers to understand better the function of such candidates in the diseases of interest.

In the short and medium term, this programme of work will benefit the pharmaceutical industry. Target validation in rodent models with well characterised compounds will facilitate the decision-making process within the industry. The aim is to progress within our Institute to the point of generating in vivo proof of efficacy and then partner with companies for further development. Several collaborations are already in place in early projects, where pharmaceutical companies have made available their compound libraries to drug development tailored to our institute for early screening and hit compound identification.

In the long term we anticipate that success may bring benefits to patients. The road to bring new drugs to the clinic is long in neurodegenerative disease, and there are many failures. We anticipate that this program of work will enable us to build robust strategies to facilitate the translation of compounds /targets to the clinic. Our experiments will demonstrate the effects of drugs on living organisms and how efficient they are. In turn, this will facilitate the further development of that given drug either within the institute or within a pharmaceutical partner.

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

Our team analyse various aspects of brain health in rodent models of disease, delving deeper to understand disease mechanisms and the pathways involved. We use that information to uncover targets for potential new medicines. We collaborate with a wide network of specialists beyond our group, inside and outside academia, allowing comprehensive analysis of unexpected findings, even beyond brain-related organs. All discoveries, including both positive and negative results, are shared through various channels, including publications, presentations, and media. This open approach aims to maximize knowledge dissemination and potentially accelerate therapeutic development. By transparently sharing all our findings, we hope to lay the groundwork for future breakthroughs in this field.

Species and numbers of animals expected to be used

- Mice: 10000
- Rats: 1600

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 support both the ex vivo work and in vivo testing, breeding colonies of both genetically modified and wild-type animals will be maintained. Wild-type rats and mice will be used at embryonic, neonatal and adult stages for ex vivo analyses and primary tissue culture. Adult animals will be used to clarify what the drug will do to the body and what the body does to the drug once in the system. Genetically modified mice will be sourced from existing colonies or bred in house where appropriate. These lines will have mutations that cause the animals to develop key hallmarks of the specific neurodegenerative disease. or will have modified expression of proteins involved in specific biological pathways related to neurodegeneration. Due to the nature of our studies, focusing on the biology of neurodegenerative disease, some of the animals used in the studies will be aged. This is because the level of the protein and/ or disease phenotype only appears at later stages of adulthood in some of the rodent models used. Throughout our studies, animals will be assessed for their welfare by following the UK's National Centre for Replacement, Reduction and Refinement (NC3Rs) guidelines (physical, behavioural and psychological wellbeing).

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

Typically, genetically modified adult animals which have some phenotypic symptoms of

neurodegenerative diseases will be dosed with a drug that we have developed, and the therapeutic effect of such treatment will be assessed by our team. We will evaluate how efficient that drug is in improving the signs and symptoms of the disease (Alzheimer’s disease (AD) or a similar neurodegenerative disease). In terms of AD for example, we will evaluate whether the drug reduces inflammation in the brain and if the progress of the disease is altered or delayed by the treatment. We will also extract fluids from the animals (spinal fluid via lumbar puncture and /or blood typically via tail vein sampling, using a single use needle) and check for biomarkers, which are indicative of the progression of the disease. The number of procedures will depend on the regimen of the drug – we always aim to develop drugs that require the least number of administrations, and therefore minimise the number of procedures.

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

The likely maximum level of severity is moderate and most procedures will be mild.

Following injections, animals might experience transient mild pain, but more likely they will not experience any adverse effect.

In the unlikely event of a surgical procedure (implantation of a canula to deliver the drug) some animals might experience moderate pain and/or distress, but that should not last more than a few hours.

Anaesthesia and analgesia will be established and maintained at sufficient level for the animal not to feel pain throughout the entire procedure. Analgesia will be given to manage any possible postoperative pain.

Following any procedure, all animals will be observed by trained staff to check for sign of distress such as:

appearance

behavioural abnormality or distress

weight loss

alteration in their natural behaviour and interaction with other animals.

Appropriate remedial action will be taken, and the following endpoints used when necessary to limit suffering: where anaesthesia is required, animals will be monitored and housed separately from others until fully recovered; more frequent monitoring; placing on a heated blanket; if the condition deteriorates and/or the severity is likely to exceed moderate, 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)?

For the majority of the mice bred on this licence, their genetic alterations will produce mild or slow developing symptoms (e.g. typical AD cognitive symptoms), and hence their severity is not expected to exceed the mild limit.

In addition, animals (wild type and transgenics) may also experience moderate severity if they have undergone procedures to investigate the efficacy (the ability of a drug to produce a desired effect) of a therapeutic treatment on disease progression.

Overall, across all the protocols, we expect the following:

Mice

Subthreshold severity: 10% Mild severity: 60%

Moderate severity: 30%

Rats

Subthreshold severity: 10%

Mild severity: 70%

Moderate severity: 20%

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?

This project requires the use of animals for several reasons. Firstly, an intact system is required to assess the pharmacokinetic properties (absorption, bioavailability and metabolism - ADME) of a molecule. This is critical for translation into efficacy and safety testing for potential novel therapeutics. Secondly, neurodegeneration is a complex and multifactorial process. The ultimate aim of this programme of work is to translate basic biological findings into in vivo efficacy and ultimately – if successful - to underpin clinical translation. For this programme of work, we will use replacement where possible and undertake in vivo experimentation only when we believe that we have the possibility of generating meaningful data. We will only use a compound for an in vivo study if its potency and in vitro ADME data suggest that it might have sufficient exposure.

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

A number of projects are supported by computational medicinal chemistry. Predictive models of offtarget toxicity can be used to guide the selection of specific ADME studies to confirm the model and triage compounds. We will also use – where there is a scientifically meaningful cell model to use – native in vitro assays using cell lines, to confirm primary potency from initial screening assays. We will also utilise commercially available humanised stem cells, for example, to replace the use of animal tissues where available. We already have collaborations with several labs across the University we are based at, a specific type of cells which have the ability to develop into any cell type in the body. Based on current projects, >99.5% of all compounds made are not progressed into animal studies.

Why were they not suitable?

These alternatives are continuously used in our group. Whilst they are suitable and informative they are limited, as we need to understand pathways and interactions among different cells and tissue in a complex model system, which cannot always be recapitulated in a dish. Furthermore, unfortunately science has not yet advanced to the stage in which we can grow brain in culture as it is one of the most complex systems in our body.

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 predicted based on the number of animals used in the past 4 years and forecasted for the activities planned for the next 5 years in the institute. Since the new Chief Scientific Officer, was appointed in 2022, there was a significant increase in the number of activities within the institute and number of projects in our portfolio almost doubled. Further, many of the projects are now at the lead to optimisation stage of the drug discovery development phase, when the in vivo experiments start. Based on the above, we made a conservative prediction for the number of animals required for this licence. We calculated this estimate considering our group's historical research output, the anticipated number of researchers involved throughout the project, and the statistically significant number of mice required for our experiments.

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

Careful design of breeding strategy is important to minimise the number of unproductive crosses and the fraction of unwanted offspring. In addition, littermate controls will be used, thus reducing the number of animals not used for experiments. We will use the minimum numbers of animals possible to test the hypothesis. Our team have extensive experience designing appropriately powered experiments. Power calculations will be performed to establish appropriate sample sizes, with a required significance level of 5 %. These calculations will be based on effect size from pilot biomarker studies of positive control compounds and/or knowledge of the PK characteristics of the compound to be tested. Animals will be randomly allocated to groups and fluctuations in experimental environment minimised to control variation in studies. Factorial experimental designs will be used wherever possible when factors are not directly influenced by the target being studied. We will use statistical tools such as Graphpad Prism to evaluate the number of animals needed to reach high statistical power (greater than 80% power effect). The Experimental Design resources available on the NC3Rs website will also be used as a guide.

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

As mentioned above we always carefully plan the breeding strategy when we require transgenic animals for our experiments. In addition, all of our experiments include both sexes. We are based in the same building and work collaboratively with many academic groups, we therefore tend to share resources with them (tissue, excess of cells when culturing for in vitro experiments, etc.) to avoid waste and optimise the usage of samples derived from animals. Furthermore, when planning an experiment, we tend to run initial pilot studies so we can successfully detect flaws in the study design, which may impact its reliability and validity and predict more precisely n number for bigger studies (when performing power calculations).

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 within this programme of work will take place in mice. This species was chosen as the bulk of knowledge regarding in vivo mechanisms of neurodegeneration has taken place in genetically modified mouse models. We will select the most appropriate mouse model to assess a given biological target based on the expression and activity of the target in the model. The mouse model will be chosen to answer specific questions about mechanism and must be the most biologically relevant model whilst minimising the potential adverse effects. Considering that protein aggregation in the brain is a key pathological mechanism in many neurodegenerative disorders, including AD, we will use models in which AD-relevant proteins (i.e. amyloid beta and tau) have been shown to abnormally aggregate. In such models we would test, for example, modulators of amyloid beta and/or tau production or aggregation.

No protocols within this programme of work will be categorised as severe. There is a risk of adverse effects when testing any new pharmacological agent. To mitigate this risk, single dose and possibly multiple dose pharmacokinetics studies (the study of how the body interacts with the drug) in small numbers of animals will be used prior to larger studies. During these pilot studies, animals will be monitored – if they show signs of distress then steps will be taken to try and revive them (e.g. placing on heated blankets). If these fail, animals will be humanely killed.

This project involves the use of genetically modified models either displaying neurodegenerative pathology or having mutations in key signalling pathways genes involved in neurodegeneration. It is not planned to internally generate any novel lines with unknown phenotype during the duration of this project – all genetically modified lines used will be generated and phenotyped elsewhere. Throughout the life of the animals, the effect of the genetic mutation will accumulate and consequently generate some of the pathological phenotypes observed in AD and other diseases. We will ensure that animals are monitored, and experiments are undertaken prior to the animals exhibiting symptoms that would be categorised beyond moderate.

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

The research carried on at our institute focuses on tackling dysfunction in the mammalian central nervous system, more specifically neurodegeneration and the immune response associated with it. While rodents differ from humans in many ways, their evolutionary closeness to us ensures a high degree of similarity in fundamental cellular pathways and genes . This is a key advantage over nonmammalian and more simplistic models, where these mechanisms might diverge significantly.

In addition, there is vast array of genetically modified mouse models available within our research community, recapitulating many aspects of the diseases we are interested in and allowing us to investigate and target the pathways which are involved in these diseases. This builds upon the extensive knowledge base established through decades of successful research using such models in this field. Furthermore, while many aspects of the central nervous system are conserved from invertebrates to less sentient species and to humans, lower-order organisms do not harbour the same complexity and intricacies present in the mammalian nervous system, particularly when it comes to studying the central nervous systems and interactions with the immune system in the context of neurodegeneration.

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

For all protocols it is important that the animal has no additional stress, as in addition to the welfare of the animals, it can have a significant impact on the outcome of our experiments. Therefore, animals are always handled by experienced operatives only, calmly and as standard procedure within the animal facility, tunnel or loft handling or cupping are used to decrease the level of anxiety in the animals.

All surgery will be undertaken in full compliance with Laboratory Animal Science Association aseptic technique guidance to minimise infection risk. Animals that have had anaesthesia have extra monitoring until fully recovered and extra checks when back in their home cages and holding rooms. When general anaesthetics are necessary, the combinations with least adverse effects will be used.

Pain from tail bleeds is reduced by using local anaesthesia.

Technical refinements, such as improvements on drug administration/ routes, will be developed and adopted throughout the life-time of the project and disseminated to other researchers at the Institute.

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

All routes and volumes of administration of substances and surgical procedures (aseptic techniques) will be conducted following the BVAAWF/FRAME/RSPCA/UFAW group on refinement and the LASA guidelines respectively. Experiments will be designed with the assistance of the NC3Rs Experimental Design Assistant, GraphPad Prism or with readily available online experimental design assistants/ power calculators (e.g. http://biomath.info/power/) and we will adhere to ARRIVE2 guidelines in publication and communication of experimental outputs. Our laboratory and institution conform to the highest level of quality and welfare control on all fronts, including husbandry, phenotyping and administrative processes.

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

We work very closely with the Biological Services Unit (BSU) staff who are always helpful in presenting us with innovative refinement techniques that are constantly being updated. In addition, at the university we have direct access to all of the NC3Rs resources and their local Training and Engagement Programme manager who is happy to meet and keep everyone updated on the latest on refinement approaches. Further, our BSU staff and managers are always open to implement new techniques that are beneficial to the welfare of the animals and therefore, will have a positive impact on the science.

50. Animal Models of Human Disease

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

Key words

Respiratory disease, COVID19 ARDS, Fibrosis, Pneumonia, Genetic disorders

Animal types	Life stages
Guinea pigs	Juvenile, Adult
Rats	Neonate, Juvenile, Adult, Pregnant adult
Mice	Neonate, Juvenile, Adult, Pregnant adult
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 objective of this project licence is determine how the body processes potential new medicines and whether these new medicines are a cure for 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?

It is important to perform this work because there are still many human diseases (e.g. Alzheimer's, lung fibrosis) for which there are no cures or because the cures that exist are not effective.

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

The outputs from this project licence will include the following;

- Study reports which describe how the body reacts to the new medicine and whether the new medicines are likely to be effective if given to human patients.
- Peer-reviewed publications
- Data presentations at conferences/internal meetings
- Blogs/webinars (internal and external)

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

In the short term, we will provide clients and sponsors with data and reports which help to establish if a new medicine is able to reverse disease and/or whether a new drug, given for example by intravenous infusion, reaches the target or diseased organ of relevance eg. lung, liver etc.

In the longer term, our studies form part of a much larger program of research that enables the development of successful medicines that benefit society through diagnosis, treatment or prevention of disease.

Identification of toxic reactions can prevent future harms to human volunteers or patients by resulting changes to drug development programs.

The wider scientific community will benefit from publication of data demonstrating improvements in study conduct that improve the relevance of the animal model or lead to refinements and reductions in animal usage.

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

- In-house collaborations and information exchange with others within the organisation worldwide, identifying successful and unsuccessful approaches.
- Collaboration with clients (knowledge gained on products).
- Hosting scientists and animal carers from other establishments, including universities, to promote best practice.
- Presenting outputs at international scientific conferences .The project licensee also liaises with local universities to share best practices.

Species and numbers of animals expected to be used

- Mice: 15,500
- Rats: 11,000
- Guinea pigs: 600
- Rabbits: 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.

New medicines are approved for use in human patients by governmental bodies (e.g.Medicines & Healthcare products Regulatory Agency) who authorise them as safe for the treatment and effective cures. The animal types used in this licence are recognised by regulatory bodies as providing information to help select a safe cure for human diseases.

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

Many of the studies conducted in this licence require that lung injury is induced which leads to inflammation and sometimes fibrosis (gas exchange region of the lungs). Animals will be administered with different agents to cause lung injury on one or more occasions. The process of administering agents to the lungs will be performed under anaesthesia and therefore the animals will not feel any discomfort from the process.

Animals will then be treated with potential cures given by a clinically relevant dose route e.g. by mouth, by infusion into the blood or by inhalation to the lungs. All these dose routes are expected to cause only momentary discomfort. For injections into the blood, the injection site will be alternated to reduce discomfort. Treatments may continue once or twice daily for 1 or 2 days (e.g. short term studies) or for one month (e.g. longer term studies investigating fibrosis).

Animals maybe monitored during development of the disease using methods to assess shortness of breath; in this process, animals are placed in a small chamber and measurements taken. The animals are not expected to feel much discomfort during this respiratory assessment since they are free to move in the chamber. Generally, when they are first placed in the chamber they are investigate their surroundings (10 to 15 mins) and then fall asleep. However, it may also be necessary to asses breathing using a system in which the animals are held in restraint tubes and may feel some temporary discomfort. To reduce discomfort, the animals will be acclimatised to the restraint tube and offered a treat after the procedure. On average this procedure takes approximately 30 mins and the may be performed once on a short term study or a couple of times on a longer term study.

Other study types on this licence will investigate the development of new cough medicines and in these studies animals will be induced to cough for periods up to 20 mins one one or a couple of occasions. Animals may feel discomfort during the coughing period (about 20 mins) but soon return to normal once the coughing has subsided. Animals may be induced to cough a couple of times over a 2 week period; in our experience, repeated coughing has no detrimental effects.

Other study types are designed to investigate how new medicines are processed by the body animals. In these study types the new medicine will be administered by a clinically relevant route of delivery (e.g oral) and blood samples taken to assess drug levels. As described above, animals may feel a momentary needle-stick pain for blood sampling,. Typically, these types of studies take place over a 24h period and the animal exhibits normal behavior for the duration of the study.

This license is also designed development new treatments for lung cancer. In this respect animals may experience the same symptoms as cancer patients ie. muscle wasting and respiratory discomfort.

Some studies may be undertaken to assess severe acute lung injury as occurs in patients who have had major trauma or COVID19. In these studies, animals will be anesthetised and put on artificial ventilation from the start of the study and will therefore not feel any pain or discomfort.

In addition, some studies are designed evaluate new medicines for a disease of premature babies called Bronchopulmonary Dysplasia (or BPD). This disease has ben discribed for over 50 years but there are limited treatment options. Premature babies are given supplemental oxygen - which enables the babies to survive. However, extended periods of high oxygen concentrations leads to delayed lung growth and development. This disease is modeled in newly born mice and rats by exposure to oxygen while remaining with the foster dam.

Some studies maybe undertaken to evaluate new treatments to restore smell. In these studies, animals will be given an agent that leads to localised inflammation in the nose-leading to a temporary reduction in smell. Treatments will be administered (as per other studies) to determine if they are able to restore smell. Studies will be undertaken to assess the abiity of the animals to finde good or avoid unpleasent smells. These studies will normally require monitoring the time taken for the animal to find the nice smell or avoid an unpleasant smell.

Finally, some studies maybe conducted in genetically modified mice which have signs associated with the human clinical disease for which there are no cures or limited cures.g. Duchenne's, Huntington's. New treatments will be given by animals to determine if the symptoms can be reversed and show the treatment does not cause other side-effects.

For all the studies conducted, animals will be made familiar with the process in 'dry runs' so that they become accustomed to the procedure prior to the actual test. Animals will also be given treats (e.g.

rice pops or pumpkin seeds) whenever possible after a procedure.

At the end of all studies in this licence, animals will be humanely killed under terminal anesthesia.

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

Previous experience has shown that most animals with lung injury lose their appetite leading to weight lose. Animals also develop shortness of breath as observed by an increased rate of breathing. These changes are generally seen during the first few days of a study and gradually decline in longer term studies. Animals are supported in this phase by being offered high energy food supplements (e.g. Complan) to encourage weight gain. Also, the animals are very closely monitored during the study and charts are generated on a daily basis to monitor individual animals and humanely kill animals who exhibit other signs. In longer term fibrosis studies, the animals recover and are indistinguishable from normal animals.

In animals who develop lung cancer the symptoms will progress if untreated therefore the study will be run for the shortest period possible to allow assessment of the new treatment.

Animals with lung cancer will be provided with food supplements to encourage fluid and food in-take.

In studies designed to evaluate new cough medicines, animals may cough for a 20 min period and therefore the extent of discomfort is expected to be temporary.

In studies designed to evaluate new treatments for the restoration of smell, the animals will have reduced/temporary loss of smell for a short time.

In studies designed to investigate BPD newborn pups - we expect the pups to have minimum discomfort based on outward signs of general growth and normal behavior (ie remain with the dam and continue to feed normally).

In studies designed to investigate blood levels of a new medicine, the animals may feel mild discomfort during blood sampling but will otherwise act normally.

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)?

Using data from Home Office returns the following is expected based on the use of rats and mice;

Mild: Approx. 25%

Moderate: Approx 75%

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?

While non-animal methods are useful to identify test item effects on individual cell types and on individual chemical pathways, they are currently not able to predict effects on whole body systems or to provide information on how a body reacts to a new medicine. Therefore, it is not currently possible to acquire all of the information on how the heart, brain and lungs may be affected by new drugs, without using animals. This information is essential, to protect human volunteers and patients. Although the studies conducted in this project license are not governed by regulatory authorities (e.g. MHRA) it is generally accepted by scientists that non-animal alternatives are not able to predict the dose in humans of a potential new medicine.

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

Our laboratories, are developing and validating new in vitro assays to assess toxicity using human cells. However, these in vitro systems currently do not mimic all the processes that

happen in the body. Therefore, it not yet safe to predict the safe dose of a medicine based on the results from nonanimal alternatives. That said, we are able to use complex human in vitro lung models to assess aspects of dose formulation (e.g. safety to the lung) prior to working with animals and thereby reduce and refine the animal studies.

Why were they not suitable?

There currently remains general scientific agreement that to protect human volunteers and patients, non-animal alternatives do not, as yet, provide enough information to replace animal studies. That said, the use of non-animal alternatives is progressing at pace and while not yet approved for deciding if a drug is safe - these new non-animal methods are being used instead of animals at various stages along the drug development pathway. We recommend the use non-animal technologies to support drug development.

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 estimates are based on analysis of use of animals in an existing licence authorising work for the same purpose, combined with anticipated upcoming studies.

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

Statisticians have provided advice on experimental design for all standard study designs within the project. This ensures that studies are correctly designed to meet the study objectives. Statisticians will be consulted on a case-by-case basis for any study that requires a non-standard design.

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 investigate the potential of new designs to improve outcomes. These could include evaluation of new ways to analyse the data, or additional tests to conduct, leading to improved data quality and translation to humans.

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.

Rats, mice, guinea pigs.

We use the above species because they develop aspects of the human disease in relatively short-time frame (e.g. days to weeks) that can be improved by known cures (if available) (e.g. antibiotics for pneumonia, codeine for cough).

Individual animals are carefully monitored during the study in the form of daily observations. Charts are prepared for each individual animal so that welfare is closely monitored after treatments and the study design modified to reduce any harm (e.g lower dose of the new medicine).

Potential medicines are given to animals by common routes (e.g. in food, by mouth, by IV); all these methods are well established and common methods for the species to be used.

Volumes of potential medicines given to animals are in-line with published guidance on minimising discomfort, and/or are known to cause minimal discomfort based on extensive experience at the site.

Blood sampling follows published guidance on suitable volumes which can be taken while minimising harms to animals.

Collaborations are currently on-going to determine whether improvements in disease assessment could be implemented to reduce the study time from weeks to days in some models.

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

The species used are selected based on known standards of outcome which will answer the scientific questions.

To enable comparisons with other data being generated in the same species as part of the safety assessment of potential new medicines.

The use of terminally anaesthetised animals would not model human disease and treatment (apart from the model of Adult Respiratory Distress Syndrome which is being performed in terminally anaesthetised animals).

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

- Monitoring of on-going procedures will be refined as any causes for concern are identified; for example, reduced dose of a challenge agent.

- The surgery and anaesthesia/pain relief protocols will undergo continual assessment and refinement to improve outcomes.

- Continue collaborations to improve *in vitro* assessment (to better predict safe doses to humans)
-

Continue collaborations to assess the effectiveness of a new cures in days rather than weeks and therefore reduce the length of studies and number of procedures per animal.

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

Following guidelines from relevant working group publications (e.g.An Official American Thoracic

Society Workshop Report: Use of Animal Models for the Preclinical Assessment of Potential Therapies for Pulmonary Fibrosis, 2017).

Dose volume and blood volume limits agreed with the animal welfare and ethical review body are based on the 2001 publication of Diehl et al: A good practice guide to the administration of substances and removal of blood, including routes and volumes.

Welfare end-points are developed in general line with publications on the topic, including the NC3Rs document from 2010 on dose level selection for regulatory toxicology studies.

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

We are involved with other groups working in the same field, and have participated in analysis and publication of data in the scientific field to assess methods of conduct and outcomes. This establishment has been involved with various working groups of the National Centre for the Replacement, Refinement and Reduction of Animals in Research over many years.

51. Targeting proteins involved in nucleotide synthesis in T-cell Acute Lymphoblastic Leukaemia

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

Acute Lymphoblastic Leukaemia, nucleotide synthesis, drug target validation

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 aim of the proposed research is to expand human T-cell acute lymphoblastic leukaemia (T-ALL) patient samples in mice, which enables us to obtain a large number of live cells. From these we will isolate proteins, which we will use to determine the expression levels of the amino acid transporter EAAT1 (Excitatory Amino Acid Transporter 1) in human T-ALL 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?

T-cell acute lymphoblastic leukaemia (T-ALL) is a severe illness that affects around 100 children and 100 adults each year in the UK, with about 100,000 cases worldwide.

Roughly 50% of adults and 15% of children with T-ALL do not respond to current treatments or relapse. Unfortunately, there are no effective alternative treatments for these patients, and most will not survive. This makes it crucial to find new treatment options.

Using T-ALL cell lines as a model, we identified a protein that is crucial for T-ALL proliferation and survival. This protein is called EAAT1 and is normally only expressed in the central nervous system. Having investigated samples from patients, we know that 95% of T-ALL patients express the mRNA (the genetic information to produce protein) for EAAT1. Having identified that EAAT1 is essential for the survival and proliferation of the T-ALL cell lines, it means that the EAAT1 protein is a potential drug target for the treatment of T-ALL.

Whilst this is a potential target, we know that mRNA levels vary between patients and this does not necessarily correlate well with protein levels that are expressed. It is important to establish what proportion of patients express sufficient protein to make this a viable therapeutic route.

The limiting factor is that T-ALL is a rare cancer and so we rely on an archive of frozen patient samples. Because (1) the amount of cells collected from human donors is relatively small, (2) samples are of varying quality due to freezing conditions, and (3) the EAAT1 levels are very low, we need to use mice to grow the leukaemia samples sufficiently to isolate and analyse the amount of EAAT1 that is being expressed. Once we have expanded the samples in mice, we will have sufficient material to measure the EAAT1 protein levels and deduce the level of expression in the original T-ALL patient samples.

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

At the end of this project we will have sufficient material originating from patients' samples and expanded up in mice in order to determine the amount of EAAT1 protein expressed in the original samples. This will enable us to address the question of whether targeting of EAAT1 is a viable therapeutic option for T-ALL patients.

This new information will support the future development of EAAT1 inhibitors (and associated patents) and will be incorporated in scientific publications, which will share our findings with the scientific community.

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

The findings from these experiments will show the presence of EAAT1 in human T-ALL samples. Confirmation that the expression of EAAT1 protein is in line with our in vitro data and published mRNA expression of EAAT1 in patient samples will strengthen the position of EAAT1 as a potential drug target.

In the short term, the data will provide baseline information as to how impactful EAAT1 inhibitors could be in the treatment of T-ALL.

In the medium term, the strengthened data will enable us to set up collaborations with those who could aid the development of EAAT1 inhibitors. Additionally, through discussions this will benefit scientific collaborators and the results will be disseminated through scientific publication in high impact journals and presentation at scientific meetings, which benefits collaborators, colleagues and other members of the field.

In the longer term, we think that the above processes will result in the use of EAAT1 inhibitors as a means to treat T-ALL (initially refractory and relapsed disease), which would show clear patient benefit. Furthermore, we identified that this protein is expressed in a number of other cancer types such as ovarian, neuroblastoma, bladder, and head and neck squamous cell carcinomas, where in some of these it is associated with significantly shorter overall survival. Therefore, also these cancers may benefit from EAAT1 inhibition.

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

Publishing our results in peer-reviewed journals with open access ensures that new knowledge is shared widely and quickly. We will also present our findings at scientific conferences. If any of our experiments suggest that EAAT1 is not a suitable drug target, we will share these results too, for example, through open-access platforms like BioRxiv.

Species and numbers of animals expected to be used

- Mice: 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.

In order to study tumour progression we will use immunocompromised mice. This is required because wild type mice would reject the cells, so tumour formation would not occur. Due to the requirement to inject the cells into the mice and the time it takes for T-ALL development to progress, we need to use adult mice.

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

Animals will be injected intravenously with human tumour cells to start engraftment of leukaemia cells. The leukaemia is then allowed to develop for up to 16 weeks. We will monitor through blood sampling how the T-ALL development responds. At the end-point of the experiments the animals will be humanely killed using a schedule 1 method.

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

During the early experimental stages the majority of mice will experience mild discomfort caused by the administration of injections, and/or monitoring of tumour burden through blood sampling. The time it takes an animal to fully develop leukaemia will vary, depending on the cells that are engrafted. Primary patient samples are estimated to take up to around 12 weeks to engraft. By monitoring leukaemia development, where possible we aim to end experiments before the animals exhibit adverse effects, however, some animals may display signs such as abnormal breathing patterns, an abnormal gait, intermittent diarrhoea, loss of body weight (up to 15%), or loss of body condition (animals are not permitted to drop to 1 on body condition scoring, where 2 is considered a normal animal). For this we will have a scientific end point of the detection of >25% human CD45+ cells as a percentage of peripheral blood mononuclear cells.

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 is moderate, where animals with full tumour development are the ones that will experience the moderate classification. For this reason, 100% of animals may experience this severity, although by monitoring leukaemia development we will aim to end experiments as early as possible whilst still achieving our scientific aims.

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 work is based on in vitro work using cell lines. In order to confirm that our findings, relating to the function of proteins in leukaemia development, are also relevant in vivo in patient samples, we require to determine EAAT1 expression levels in human T-ALL cells.

Immunocompromised mouse models are most appropriate for engrafting human T-ALL cells and to maintain their original characteristics. We can then isolate these to determine protein expression.

Alongside the animal work, and using the protein samples from the mice, we are developing assays (ELISA) for patient-derived samples with the intention of improving sensitivity. If successful, this will remove future need to engraft and expand tumours in mice in order to measure EAAT1 protein levels.

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

We always perform experiments first using in vitro cell cultures with cell lines and/or primary cells. Unfortunately, cell lines do not fully represent samples taken directly from patients and patient samples cannot currently be expanded in vitro. Human T-ALL xenografts in mice is the approach widely used and published for expanding primary T-ALL samples.

Why were they not suitable?

Unfortunately, cell lines do not represent primary patient samples and patient samples cannot currently be expanded in vitro. This is likely due to various aspects of tumour biology, such as tumour microenvironment and physiological homeostasis.

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 defined how many mice we need to engraft for each patient sample; to ensure successful engraftment without using more mice than required. This is based on data obtained by colleagues under similar experimental set ups. We aim to determine EAAT1 protein levels in approximately 20 TALL patient samples within this project.

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

The use of methods to monitor the leukaemia development, such as blood sampling, will result in less variability as animals are their own longitudinal control and avoids the need to humanely kill animals to measure disease progression.

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 point we will collect as much data and tissues as reasonable, which can then also be shared with other research 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 immunocompromised mice. These are the model used for leukaemia engraftment as they do not reject the tumour cells. There are no established alternative models in which we can recreate the tumour microenvironment and physiological homeostasis.

Although there is no alternative approach, we ensure that the model is as refined as possible. We will closely monitor the animals undergoing procedures using score sheets, especially towards the further development of the tumours. Tumour burden will be kept to a minimum within the requirements for the experiment. By monitoring disease burden through the use of blood sampling, wherever possible we will terminate the experiment before the animal develops signs of stress and discomfort.

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

In mice, leukaemia engraftment and development can only be performed in the adult stage. Tumour progression takes time as well. Therefore, we cannot use immature mouse stages that are less sentient, nor can this happen under terminal anaesthesia. As we require a functional tumour microenvironment and physiological homeostasis for the human T-ALL cells to develop, we need a mammalian system and cannot use less sentient species.

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

We will closely monitor the animals undergoing procedures and especially towards the further development of the tumours. Tumour burden will be kept to a minimum within the requirements for the experiment. By monitoring disease burden through the use of blood sampling, wherever possible we will terminate the experiment before the animal develops signs of stress and discomfort.

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

We will use the guidelines set out by the Laboratory Animal Science Association (LASA), particularly those relating to injection route/volumes and blood sampling, as well as the PREPARE and ARRIVE guidelines where appropriate.

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

We will stay aware of any advances related to the 3Rs through (1) information available from our licenced establishment, (2) keeping up to date with related research publications, (3) the NC3R website, which contains information as well as on line tools, (4) other information sources, like other research groups.

52. Manipulation and evaluation of the Tumour Immune Microenvironment for Cancer 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
    - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants

Key words

Cancer, Immunology, Immunotherapy, Liver Cancer

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 project aims to enhance the fundamental understanding of how the immune response is regulated against cancer as well as the development of novel anti-cancer 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?

Cancer is one of the leading causes of death in the UK and the world. Understanding the role that the immune system plays in protecting us from cancer has recently led to the development of novel immunotherapies currently in the clinic. However, these immunotherapies only work in a fraction of patients, and much research is needed. We are particularly interested in the following types of cancer:

Liver cancers: are the leading cancer type, increasing in incidence and mortality in the UK.

Immunotherapy works in a small fraction of patients, highlighting the need to develop new treatments.

Our group will investigate fundamental immune-regulatory mechanisms controlling its development. We aim to understand its carcinogenesis (the process by which normal cells are transformed into cancer cells) and how this disease develops using mouse models.

Sarcomas: are rare tumours but of high importance as some of them affect children. We aim to use mouse models to model this disease and test new treatments, particularly vaccines.

Basic immune mechanisms driving cancer development: Besides the tumour types described above, we also expect to answer some fundamental questions using subcutaneous and lung tumour models. This includes metastasis (when cancer cells spread from the original tumour to other parts of the body) and immunological memory formation following tumour rejection.

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

Publications: All work performed under this project will have as ultimate goal the generation of highquality publications that can be accessed by everyone around the globe.

Patents: Given the translational nature of our work, some of our findings are expected to generate knowledge that may lead to new treatments, which will be registered for use in the pharmaceutical industry.

Cancer vaccines: This project will focus on developing novel cancer vaccines to prevent liver cancer in high-risk patients. These vaccines will be initially tested in preclinical liver cancer and liver fibrosis mouse models.

Novel biotherapeutics: Our work will focus on developing novel biotherapeutics (treatments that use biological substances such as antibodies and cell therapies) to fight solid tumours (cancers that form in organs or tissues).

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

In the short term, the primary beneficiaries are the scientific community:

Publications will help other researchers understand how the immune system and new targets relate to cancer

Identification of novel ways to target and treat cancer, particularly liver and brain malignancies.

Generation and preclinical testing of novel biotherapeutics for cancer (vaccines, antibodies, cell therapies)

In the long term, we expect that the primary beneficiaries of this work will be cancer patients:

1. We expect that the therapies developed using our mouse models will inform the development of novel clinical trials that will positively impact cancer patients in the future.

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

We aim to generate publications in open-access journals, present our findings at scientific meetings, and make resources (e.g., data, animals, tissues) available to other researchers. We are committed to developing a collaborative environment that will lead to new publications and patient engagement through talks.

Given the potential of our findings to be translated to other types of cancer, we expect in the future to expand towards the study of other cancer types like those that develop in bones as well as childhood malignancies.

Species and numbers of animals expected to be used

- Mice: 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.

This project will use adult mice (post weaning) to study the process of carcinogenesis (the process by which normal cells transform into cancer cells). This life stage represents the one at which liver cancers develop. We aim to replicate the long process of carcinogenesis of the liver by exposing mice to high-fat diets or alcohol, which replicate non-alcoholic fatty and alcoholic liver disease from an early age, as it currently happens with humans. Mice will either be fed with a high fat diet or alcohol from early age and then tumours will be induced, or tumours will be induced before mice go into high fat diet or alcohol consumption. For other cancer models (sarcomas), we will use adult animals as we will inject already transformed cell lines with a focus on growing tumours rather than determining how cancer cells are formed.

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

We will use genetically altered mice with a weak or disabled immune system or modified to have human cells or express human receptors. These mice allow us to assess how a treatment works when tumours are induced.

Animals will undergo induction of tumours by injecting tumour cells or fragments. Tumours will be induced in the liver, muscle (sarcomas), subcutaneous fat, or lungs. Liver tumours will be induced by injecting a limited amount of liquid containing specific substances like DNA directly into a superficial vein in the tail, or via a surgical incision to expose the liver and inject a small amount of tumour cells. Lung tumours will be induced via an intraveous injection. Tumours will be measured up to 3 times per week, usually using callipers or via non-invasive imaging in the case of tumours that grow internally (liver, lungs).

During the tumour growth period, some animals will be given substances as treatment or to induce specific phenotypes (e.g., cancer vaccines, antibodies, cell therapies, chemicals). These substances will be administered mainly intraperitoneally, intravenously, or orally.

Some animals will undergo blood sampling to assess the immune response to the substances being administered.

Some animals will receive substances that target gene deletion and allow the study of specific genes.

Some mice will be exposed to high dose irradiation to deplete their bone marrow, followed by a bone marrow transplant from a different mouse strain to restore it while in other cases we will use low doses as treatment. This process resembles human bone marrow transplantation, commonly used to treat blood and immune system disorders affecting the marrow and enables tracking of specific immune components. To prevent opportunistic infections, antibiotics may be given, typically for up to 28 days around the time of irradiation.

Some animals may undergo a second induction of tumours either subcutaneously, in the liver, or intravenously to assess immunological memory.

When studying liver cancer, some animals may undergo an alteration of their diet (high fat) to evaluate the effect of liver diseases (Non-Alcoholic Fatty Liver Disease) in the development of cancer.

At the end of the experiments, all animals will be 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?

Subcutaneous tumours might invade the surrounding skin or muscle, potentially affecting normal movement. Some tumours may ulcerate, typically presenting as a dry scab, but occasionally a wet ulcer could develop. In such cases, treatment will be administered based on veterinary advice. If there is no sign of improvement within 24 hours, animals will be humanely killed.

For animals that undergo tumour induction to the liver and lungs up to 15% weight loss is expected so mice will be regularly weighed and killed if weight loss reaches 15%. Mice will be monitored for any signs of respiratory distress and humanely killed if this is seen.

Animals that undergo a dose of sublethal irradiation will experience a temporary suppression of their immune system, making them more susceptible to infections until the bone marrow transplant starts to rebuild their immune defences. Adverse effects may include a transient weight loss of up to 20% between days 7 and 10 after irradiation, which typically resolves by day 14. Animals will be supported with moist, palatable food to ameliorate the weight loss during this time. Animals will be humanely killed if they reach 20% weight loss.

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: 77%

Mild: 23%

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 animal models to understand how the immune response against cancer is generated and to test new therapies. The immune response to cancers happens within the tumours, which are very complex structures with many different cell types interacting and communicating with each other. In addition, immune cells within tumours are in constant movement which allows them to interact with different cells at different stages of their development. These processes are so complex that current in vitro systems are unable to fully replicate them.

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

We currently use small pieces of tumour tissue taken from patients, which we can keep alive from some time. These tissues then allow us to test different treatments on actual human tumours in the lab. Furthermore, together with industry partners we will aim to develop organ-on-a-chip systems that can partially recapitulate human tumours. These are 3D tissue cultures that preserve specific tissue functions and replicate the human physiology.

Why were they not suitable?

They are not able to fully recapitulate how the immune system responds to tumours. Moreover, they are not suitable for long-term studies or the understanding of carcinogenesis (how cancers are formed and normal cells transformed into cancer cells). However, these methods are complimentary and can help reduce the number of animals used.

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 collaboration with a statistician, we have made calculations using typical variations from our own earlier experimentation to define the minimum numbers of animals needed whilst ensuring that the results are statistically significant. Sample sizes for our experiments are estimated from past experiments. Calculations typically show that we need group sizes of 5 per experiment to achieve the quality of results we need. We've used previous annual return of procedures from collaborators 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?

NC3R's Experimental Design Assistant (www.nc3rs.org.uk/experimental-design-assistant-eda)

PREPARE guidelines – (https://norecopa.no/prepare)

ARRIVE guidelines (www.nc3rs.org.uk/arrive-guidelines)

OBSERVE guidelines (https://doi.org/10.1038/s41596-024-00998-w)

WORKMAN Cancer Guidelines

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: Colonies will be managed according to best practice guidelines. Particular attention will be paid to genetic stability and good breeding performance. Data from breeding animals are readily available from an in-house database and will be used to make decisions on future breeding stock and to assist in maintaining a suitable colony size to ensure only those animals needed for experiments are produced. Cryopreservation of colonies that are not required in the short term will be considered.

Pilot studies: To reduce the number of animals used, we will perform pilot studies to set up techniques and models. This will allow us to optimise models (tumour growth, time of treatment, etc) before using larger numbers of animals.

Computer modelling: We will use cutting-edge computational techniques to assess the statistical significance of our findings.

Sharing of tissues and cryopreservation: When possible, tissues will be shared with other researchers to validate our techniques and cryopreserve for future 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.

We will use mouse models due to their genetic similarity with humans and the current availability of multiple optimised transgenic and tumour models that allow for accurate refinement and reduction. We will use adult mice as they allow us to study carcinogenesis during tumour development. In some cases, we will use genetically altered mouse modes to induce tumours or to evaluate the role of specific subsets of cells. These models do not show any adverse effects prior to tumour induction.

We will induce tumours via, subcutaneous, intravenous, intramuscular or intrahepaticinjection. We will use the least invasive route when possible. We will follow best practice guidelines and monitor animals frequently to minimise suffering, distress, or lasting harm. We will anaesthetise animals when needed to better position the tumour.

Animals will undergo the administration of substances. Whenever possible, the least invasive route of administration will be chosen. If intravenous injection is needed, both lateral tail veins will be used to minimise the risk of vein damage.

Blood sampling is expected to cause only mild and transient pain.

We will use the minimal irradiating dose needed to achieve bone marrow depletion or when needed as treatment. Using two lower doses with a resting period in between also reduces animal radiation sickness.

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

Non-mammalian animals are limited in their use because they either do not have the right type of immune cell (such as Kupfer cells, T cells, regulatory T cells, dendritic cells and B cells), or their immune system is too different from the human immune system to provide relevant results (such as zebrafish). 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, which would make our results irrelevant for use in human patients.

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

Mice bearing tumours will be carefully monitored by staff trained to work with these models. Group sizes in tumour experiments will be increased to account for potential animal loss, ensuring the study remains valid and avoiding single housing. We will employ the least invasive methods possible to induce tumours whenever feasible. Model refinement is an ongoing process that necessitates careful consideration at all stages of experimentation. This commitment is instilled in all researchers within my lab. We plan to develop specific refinement procedures in our license for each individual procedure:

Liver Tumors: We will utilize established protocols from previous research and collaborate with experts who have developed these methodologies in their laboratories. Tumors will primarily be induced through injecting a limited amount of liquid containing specific substances like DNA directly into a superficial vein in the tail, which is an efficient technique. When employing orthotopic inoculation within the liver, we will adhere to established protocols from key collaborators, supplemented by our refined techniques and expertise in mouse surgery.

Sarcoma Induction: We have developed standard operating procedures for inducing intramuscular tumours in rodents. These procedures will be performed under anaesthesia, utilizing minimal injection volumes to mitigate pain. For inducible sarcoma tumours (e.g., those induced using substances), we will follow established standard operating procedures and administer minimal doses of the inducing agents.

Subcutaneous and Lung Tumors: Our lab possesses extensive experience with mouse models for these types of cancer. We will apply well-established standard operating protocols, ensuring anaesthesia for subcutaneous tumours induction and implementing short restraint periods for tail vein injections used to induce lung tumours.

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

The following guidelines will be used to train the staff as well as to check for specific questions when required:

www.nc3rs.org.uk https://norecopa.no https://www.lasa.co.uk

OBSERVE guidelines (https://doi.org/10.1038/s41596-024-00998-w)

WORKMAN Cancer Guidelines

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

We will attend 3R's events and will constantly check news and updates in the following websites:

www.nc3rs.org.uk https://science.rspca.org.uk

Finally, given the relevance of assessing human tissues, our group is committed to validating all of our findings in human tissues and working towards the development of better models and techniques.

53. Neural circuits underlying mouse behaviour

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Brain, Behaviour, Hearing, Vision, Rodent

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 understand how and where signals coming from the senses are encoded and combined in the brain (with a particular focus on vision and hearing), and how this process changes with internal state (e.g. engagement, learning) and different 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?

Combining information from different sensory systems, termed multisensory integration, is common across animals and prevalent in humans. We use lip movements to locate and understand a conversation partner, which is why ventriloquism is so effective. This process is important for healthy brain function and atypical multisensory integration has been linked to schizophrenia, autism spectrum disorder, and aging. To understand how deficits in multisensory integration cause cognitive impairments, we first need to dissect the neural mechanisms that underly this process. The latest technical and genetic tools available in the mouse model system provide an unprecedented opportunity to achieve this goal.

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

Our main outputs are scientific publications and other forms of dissemination (e.g. presentations at conferences) to enhance our understanding of the brain. Through this dissemination, we communicate new knowledge about how brains use sensory information, like images and sounds, to make decisions about the external world. In particular, we focus on how the signals from different senses, like vision and hearing, are combined in the brain before making these decisions--a process called multisensory integration. We also produce software, hardware, and methods which are made freely available and can be utilized by the scientific community to better achieve their research goals.

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

Our outputs primarily benefit scientific research and the scientific community. Our research is typically divided into projects (2-4 years), and as each project progresses we present intermediate results at international conferences. Final results are published in scientific papers, which we release via a preprint server, and submit to a journal for peer review and publication. Upon publication we make all data and analysis code freely available, as well as providing access to any new software, methods, or devices that were developed for the study.

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

Our approach is highly collaborative. We are part of an extensive community within our institution and neighbouring institutions, and collaborate with multiple laboratories to share resources and experiments to tackle complex scientific questions. For example, we are heavily involved in a collaboration between scientists and engineers to deliver transformative devices for brain recordings: by combining the surgical experiences of multiple laboratories, we can quickly converge on the optimal approach to minimise animal discomfort and maximise data quality.

We disseminate new knowledge through publications, review articles and conference presentations. We report not only on successful approaches, but also on any limitations that we discover in established techniques. We release relevant analysis code and data for all publications, and these releases can form the basis of new collaborations, or studies within other groups. Our recent technical advances in recording neural activity across longer timescales have been implemented in many laboratories across 4 continents. This exemplifies that our approach is useful to the neuroscience community, and providing multiple uses for the data we acquire.

Species and numbers of animals expected to be used

- Mice: 6,500

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.

Mammals share fundamental similarities in brain function and organisation. Therefore, to gain insights into the human brain, many neuroscientists have converged on using mice due to their rapid breeding and maturation, well-established husbandry and infrastructure, and possibilities for genetic control. These advantages, and the extensive knowledge

already established about the mouse brain, make mice the most efficient species for our experiments in terms of resources and research impact.

Furthermore, in the mouse we have access to brain activity at the level of vast populations of individual neurons, and we have tools to manipulate the underlying circuits.

We use adult mice since we are interested in studying behaviour and learning in the adult stage of life.

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

Mice (transgenic or wild type) are bred using standard procedures (Protocol 1). Some mice then receive one or more injections in the brain to manipulate selected types of neurons, e.g., to make them express some genes or to trace their connections.

Mice then proceed to behavioural / neural measurements (Protocol 2). Mice typically undergo surgery to implant a small headplate to allow them to be restrained in a stable position for recording in future experiments. In the same surgical session, we may create a transparent window on top of the skull or in the skull so that mice can undergo non-invasive optical imaging. In some of these mice, we may temporarily inactivate small brain regions during experiments using non-invasive optical or chemical means.

Alternatively, we may create a small hole in the skull to directly access the brain and administer substances or insert very fine electrodes. These electrodes may be implanted for days or months (chronically) or inserted and retracted within a single recording session (acutely).

In a small minority of mice, we may perform a small and precise cut to separate brain regions which would normally be connected, to study their causal involvement in behaviour or neural activity. For example, we may perform a callosotomy, cutting the nerve fibres connecting the two hemispheres, or a transectomy, disrupting communication between specific brain regions. These procedures do not have any observable impact on the normal behaviour of the mouse.

Whenever feasible, all surgical procedures are carried out in a single surgical session. However, they sometimes need to be split into multiple sessions, e.g., to allow for the mouse to learn a complex task.

In rare cases, and only when necessary, we perform minor repair or replacement procedures under mild anaesthesia, which are not painful as they are not invasive (for example, we may apply a little extra bonding agent). These interventions are restricted to cases where the intervention will reduce the overall harm (e.g. preventing infections) and increase the data acquired from the mouse, reducing the number of animals needed to achieve project aims.

Mice are continuously monitored throughout and after surgery, in consultation with NACWOs and NVS —particularly in the rare cases where a specific treatment plan is needed to ameliorate an adverse effect.

Some mice are trained to perform behavioural tasks with fluid or food rewards. For instance, we may train head-fixed mice to navigate a virtual corridor on a running wheel, or to turn a wheel to indicate a decision between two options. Training sessions typically last 1-3 hours and are repeated daily for 2-6 weeks, followed by weeks of steady trained behaviour. To train the mice we control their water or food intake (but not both), supplementing as needed to ensure they receive a minimum daily amount. This amount is

calculated based on the animal's weight, which is monitored daily and is adjusted to keep the weight within a target percentage of their starting weight. Before, during and/or after this training, we may record or manipulate brain activity.

Mice may need to be singly housed from the point of training or surgery if co-housing is not possible. For example, some mice will have implants that can be chewed or damaged by a cage mate, or only one mouse in a given cage may be selected for water control experiments and cannot be co-housed with mice that have free access to water.

At the end of a series of experiments typically lasting between 3 weeks and 6 months, the mouse will be killed. Depending on the scientific goal, we usually perfuse the heart and extract the brain for anatomical investigations.

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

To ensure the well-being of all mice, we adhere to rigorous protocols aimed at maintaining good health and minimizing pain. Mice will experience post-operative pain, but typically have 7 full days of recovery (5 minimum), during which preventative and post-operative pain management is provided as needed.

Most mice recover uneventfully, but a small fraction may experience transient postoperative weight

loss or minor localized bleeding, with rare occurrences of wound complications like reopening or scabbing.

Water or food restriction are well-established protocols and both are well-tolerated by mice, typically resulting in the loss of 10-15% of baseline body weight, which lasts for the duration of the control period. In rare cases (less than 5 % of mice), the weight of the mice may approach 20% of their baseline body weight, in which case they are immediately provided with supplementary water or food to increase their weight, and will be closely monitored for clinical indicators of distress, and

In a small percentage of mice, interventions to influence neural activity, such as electrode insertion or chemical stimulation, may result in localised bleeding, abnormal motor function (e.g. increased propensity to turn in one direction), or stress related to slightly prolonged head fixation.

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% Subthreshold, 25% Mild, 50% 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?

We seek to understand how the brain processes and combines different sensory inputs, how this changes across different stages of learning, behaviours, and brain states, and how this information is used to select and execute actions. To accomplish this goal, we need to study the brains of behaving animals.

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

We considered the use of computer models, in vitro assays, and human subjects.

Why were they not suitable?

Computer models are limited by our understanding of brain anatomy and physiology. Since there is so much still unknown about the brain, these models cannot yet produce the novel insights about the brain that we plan to gain through this project. We will use computer models to help us interpret animal data and generate testable hypotheses for future experiments, though these complement rather than replace animal use.

This work cannot be carried out using in vitro preparations because these preparations are vastly simplified relative to the living brain. They do not receive inputs from the sensory organs, learn from past experiences, or provide motor outputs. It is conceivable that organoids will eventually move in this direction, but even in this case, we would have little evidence that their function resembles that of the normal brain.

Finally, we cannot carry out this work on humans because human neuronal activity cannot be measured with sufficient precision or scale. Most human-recording methods, such as MRI or EEG, do not reveal the activity of individual neurons, and the very limited recordings performed during surgery only monitor a limited number of neurons in tissue that will be resected.

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 mice is based on values from other laboratories performing similar experiments. It represents the estimated fraction of successful experiments for each study and the number of mice that would constitute a reliable statistical sample for that study.

We have also conducted literature searches to confirm the typical number of mice necessary to achieve statistically significant results in these types of experiments.

We typically use about 10 mice per group.

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

We have identified reliable genetically altered strains for our work, and we will perform routine literature searches to identify new strains that could further improve the data that can be collected from individual animals.

We have consulted the NC3R's Experimental Design Assistant as a tool for planning our experiments.

The use of cutting-edge techniques to perform large-scale recordings dramatically reduces the animal numbers needed to test our hypotheses. For example, rather than recording from several different brain areas across several different mice, we can now record from several areas simultaneously in a single mouse.

Where possible, single mice are also used for multiple experiments. For example, where we seek to manipulate and record neural activity, the same mice can be used first for reversible, non-invasive manipulation, and then also used for recordings.

We have been instrumental in developing techniques to record from the same mouse across multiple experimental settings, rather than using different mice in each experiment. This greatly increases the reliability of our observations whilst simultaneously reducing the number of mice required.

We also can optimize multiple approaches in single mice (e.g. surgery strategies, task variants), which means fewer mice will be needed to reach an experimental standard to produce publication-quality data.

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.

The lab is at the forefront of developing techniques to record from large populations of neurons over long periods of time in individual mice. This not only reduces the number of mice required for our experiments, but it also produces stronger data. Indeed, recording 1,000 neurons in a single mouse for 30 days is not only more efficient but also much more powerful than recording 100 neurons in 10 mice for 1 day each, as it allows us to study how the neurons work together, and how their activity changes across time.

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 both genetically altered and wild-type mice in this project. The genetically altered lines are used to target modifications to specific cells, or connections between brain regions, and to express specialised proteins that allow us to visualise or manipulate neural activity.

The mutations in the genetically altered mice are harmless and provides the same quality of life as wild-type mice. Importantly, these genetically altered lines reduce or eliminate the need for procedures previously used to conduct these types of experiments (e.g. virus injections).

Our research depends on the mice being healthy, cooperative, and engaged in the tasks that we train them to perform, so we have many reasons to avoid any suffering. We always use appropriate anaesthetic and analgesic regimes for pain relief during surgery. Mice are allowed sufficient time to fully recover before progressing to subsequent experiments. The components we insert into the brain are typically extremely small, thin, and light. For example, the recording probes are typically 70 µm wide and 20µm thick, which is about the thickness of a human hair. After the initial surgery, these components can't cause pain while being inserted, because the brain has no pain sensitivity.

We have engineered lightweight head-holders (improved lighter titanium, which weighs on average 0.2g) and the lightest weight chronic implant to date, so that the mice are able to recover their normal mobility and activity in their home cages within a few hours of receiving these implants.

We use water or food control (but not both) as a method to motivate mice to perform tasks because it has proven to be both minimally distressful to the mice and effective for behavioural performance. Mice typically tolerate these procedures with no adverse effects, and body weight provides a robust measure of health before any more serious signs of clinical distress are observed (e.g. hunched posture or piloerection). In this way, we can increase the amount of water or food for individual mice as needed to prevent adverse effects.

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

We want our discoveries to extend as much as possible to humans, and the brain of humans has the same fundamental plan as that of other mammals, but substantial differences with that of other animals. We thus work in a mammal, the mouse.

Our goal of understanding the link between brain activity and behaviour requires wakefulness and typically also behaviour. Our recordings are thus in awake mice, rather than terminally anaesthetised ones.

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

As described in earlier sections, we constantly seek to refine our procedures. For instance, we are developing a new technique to manipulate connections between multiple different brain regions in a single mouse, reducing the need for multiple injections.

We closely monitor the health of our mice on a daily basis. For mice previously having undergone surgery, we provide additional measures for any mice with specific needs, including moist food to ensure weight maintenance, and medication to relieve pain or clean and heal wounds under NVS advice. For mice under water control, we weigh them daily and look for signs of dehydration, and increase their water or remove from water

control as necessary. Mice are habituated to being handled and head-fixed in the rigs in increasing durations before experiments begin to minimize stress.

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

To ensure best practices for refinement we will follow:

Guidance and publications from the NC3Rs and Laboratory Animal Science Association:

(https://nc3rs.org.uk/3rs-resources). In particular, "Refining the use of head fixation and fluid control in rodents" from the NC3Rs working group on high-yield rodent behaviour. For example:

- We only use dietary control and head-fixation when necessary (*points 8 & 15, recommendations for researchers*)

- Animals are taken off dietary restriction between testing periods (*point 10, recommendations for researchers*)

- Animals are habituated (*points 11 & 16, recommendations for researchers*)

- We use naturalistic stimuli, behavioural responses, and self-initiation (*point 17 & 18, recommendations for researchers*)

The ARRIVE guidelines: (https://arriveguidelines.org/)

Our protocols are informed by "Refinements to rodent head fixation and fluid/food control for neuroscience" (*Journal of Neuroscience Methods, 2022*). For example, we aim to reduce the need for water deprivation, and we minimise the duration of head-fixation wherever possible.

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

There are continuous improvements and innovations in experimental protocols and technology to reduce and refine animal numbers. We have previously operated at the cutting-edge of these methods, and we will continue to adopt any new approaches that allows us to improve in the 3Rs.

We will also engage with ongoing institutional and national 3R’s efforts, including establishment welfare meetings and 3R’s days, interacting with the NC3R’s regional manager and the Named Information Officer, and signing up to the NC3R’s newsletter.

54. Reversing the Effects of Neuromuscular Blocking Agents and Drugs of Abuse

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
- 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

Drug Reversal, Neuromuscular blockade drug, Drugs of abuse treatment

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?

This project aims to establish whether newly developed compounds collectively referred to as ‘reversal agents’ can reverse the effect of neuromuscular blockade agents and drugs of abuse (for example, opioids, hallucinogens, stimulants) by removing them from the bloodstream.

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?

Neuromuscular blockade drugs are essential for intubation during surgery; their reversal enables rapid recovery from procedures, decreasing care costs and adverse reactions.

Current methods have limitations in the kinds of neuromuscular blockade drugs they can reverse, have adverse side-effects, or prevent re-intubation. As such new methods are being developed and it is the aim of this project to validate one of these.

Drugs of abuse are responsible for >150,000 overdose deaths per year. Current methods struggle to reverse certain drug classes, including the ever more widespread synthetic opioids, which are responsible for an increasing proportion of overdose deaths. As such, new techniques for treating overdose are needed, which this project seeks to provide.

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

We are aiming to deliver data which will prove that the reversal agents can reverse the effects of drugs in animal models, helping us to understand how they can be used to create new therapeutic molecules for use in humans in the longer term. This project will also help us to understand the biodistribution of our molecule, and ensure there are no unexpected side-effects that we did not predict in cellular studies. It will also allow us to establish the appropriate dosing regime for our compounds. This project will lead to multiple scientific publications, and patentable data, including a patentable therapeutic compound. We will then seek to commercialise the most promising discoveries to maximise impact on society.

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

In the short term, the scientific community will benefit from this project due to the generation of new data and new ways of targeting drug reversal in vivo. In the medium term, those undergoing surgery and healthcare operators will benefit from new ways to reverse neuromuscular blockade drugs. In the longer term, new ways to reverse drug of abuse overdoses and the adverse behavioural effects of drugs of abuse will help substance abusers, healthcare professionals and systems, first responders, and society more widely.

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

All results of this work will be published, whether they work as intended or not, and we will maximise the dissemination of our discoveries, whilst respecting any Intellectual Property arising, by regularly presenting this work at national and international conferences. Further, we will seek to commercialise any discoveries which could have clinical impact, to maximise the output of this work.

Species and numbers of animals expected to be used

- Mice: 3900
- 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.

We will use mice and rats in this research. Mice will be used as they have many biological similarities to humans, which will allow us to confirm that our reversal agents are effective, but are the least complex mammalian lifeform that could be used. Rats will be used in limited cases where the increased size of rats are essential to generating useful data,

allowing us to directly attach electrodes to nerves. Male and female adult animals will be used in both cases, as these bear most similarity to the targeted use of our reversal agent in the adult human population.

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

We will test a maximum tolerated dose of the reversal agents in order to establish the safe therapeutic window for dosing in subsequent experiments, so that we can be sure that effects we observe are due to drug reversal, not off-target toxicity. In this experiment animals will be divided into multiple groups, where they will be given various concentrations of reversal agents and observed closely for adverse effects. Small amounts of blood may be taken to assess drug blood level and assay for any toxic effect, and other fluids may be collected. Animals will be kept for up to 3 weeks to observe longer term effects of the reversal drug and will be killed at the end of the study. If animals show adverse effects of the drugs during the study that do not subside or cannot be ameliorated they will be killed. We will monitor for signs of toxicity, both behaviourally, by weight, and by assessing fluids collected by various assays.

When we establish the tolerated dosage, we will continue with two main models.

In studies for reversal on neuromuscular blocking agents (NMBAs), animals will be anaesthetised, an intravenous cannula may be inserted into a superficial vein and 2 electrodes will be inserted under the skin of animal's leg to stimulate a motor nerve. We will record muscle twitching via specialist monitoring equipment throughout the experiment, in the presence and absence of neuromuscular blockade drugs and presence and absence of a reversal agent. We will directly compare current stateof-the-art reversal methods in parallel with our reversal agents. Animals will be anaesthetised, intubated and an NMBA delivered. When neuromuscular activity reduces, which will be observed as reduced response to stimulation of muscle with electrodes, a reversal drug will be administered (over a variable timeframe, either at short separation - 30-60 seconds - or after a longer time frame - 5-30 minutes). The reversal drug administered should increase the muscular twitch response recorded. The whole procedure will be done whilst the animal is ventilated and under anaesthesia, lasting approximately 1 hr and at the end of this experiment urine may be collected and the animal will be humanely killed without regaining consciousness.

In studies involving administration of a drug of abuse, animals will be required to go through surgical implantation of a cannula for drug administration and for blood sampling. After full recovery (at least 7 days), animals will typically be administered a drug of abuse and then one of our reversal agents, or drugs currently used in clinic to mitigate effects of drugs of abuse, or control substances. The administrations may be repeated daily for a week, with different permutations of administration. We will monitor the rate of movement of the animals in either cages that can track movement or in behaviour arenas, to confirm whether the reversal agent is able to ameliorate the effects of drugs of abuse. Small amounts of blood may be taken during the experiment to check concentration of drugs in blood. We may also collect urine to test for drug concentration. Animals will be monitored closely after drug administration until they recover normal behaviour and daily throughout the full length of the experiment, at the end of which they will be humanely killed.

If the above experiments are successful and recovery from drug of abuses using our reversal agents is confirmed, we will run experiments where animals will get an overdose of drug of abuse and then one of our reversal agents, to confirm if overdose could be reversed. For animal welfare reasons those experiments would be performed under

terminal anaesthesia where measured respiratory rate and/or heart rate would be an indicator of the reversal drug's effectiveness.

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

Studies with use of NMBA and with overdose of drug of abuse will be done fully under anaesthesia, and should not cause pain or distress. Where NMBA is used animals will also be intubated and mechanically ventilated. Procedures will be terminated and animal killed before it regains consciousness.

Surgery will be required for animals taking part in research with drugs of abuse to insert cannula where multiple daily administrations of substances are needed. Animals will be monitored for pain and discomfort and given analgesics to ameliorate those adverse effects. They will be fully recovered from surgery before they are administered drugs of abuse after which they will experience a level of abnormal behaviour (mainly increase locomotion rates) but we will minimise the duration of this effect by administering reversal agents (apart from the control group) and using the lowest dose for a detectable effect of the drug of abuse possible.

Animals used for maximum tolerated dose testing protocols of the reversal agents are not expected to experience adverse effects at lower doses of the drug but may experience behavioural changes (e.g. slow responsiveness, laboured breathing) at higher doses, which is expected to last for several hours. Animals will be observed until they return to normal behaviour and if any unexpected adverse effects are noted 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)?

Testing of maximum tolerated dose will have moderate adverse effects on the animal on around 60% of animals and 40% mild. That is approximately 6% and 4% respectively of overall number of animals used under this licence.

We will use rats in studies with NMBA. Because those studies are done fully under terminal anaesthesia, those studies are considered to have non-recovery severity, that includes 100% of animals in this type of study, and approximately 20% of the overall number of animals used under this licence.

In studies involving drugs of abuse, we estimate that all animals will reach moderate severity as they will require a surgery to implant cannula and may experience multiple drug dosing. That is approximately 45% of overall number.

All the animals in experiments with overdose of drugs of abuse will reach non-recovery severity, this accounts for approximately 25% of overall number of animals used in this project.

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 will need to use animals to achieve the aim of our project as we will have proved our technique works in in vitro assays before starting any animal work. As we are looking to develop therapeutic molecules for use in humans, we cannot progress further towards human trials without first confirming safety and efficacy of the reversal drug in animal models. In addition, the effects we seek to measure are complex, including the reversal of behavioural effects of drugs of abuse, and reversing the effect of a drug that acts to block the signalling pathways between neurons - how messages get from the brain to the muscle. This is not possible to fully explore in non-animal models. Our hope is that in the future our reversal compound could be applied into the clinic use. In order to do this, we need to test it in mammalian species. Frogs, fish and non-vertebrates would not be able to provide us with fully relevant confirmation of our compound efficiency as they lack a mammalian nervous and neuronal system, and have limited applicability on the behavioural and nervous system effects we are interested in. As we are looking to model how the Reversal agents in the bloodstream affect neuromuscular junctions and receptors in the brain across multiple biological barriers, species where modes of administration and reaction to anaesthetics and other drugs differ drastically from humans (i.e. zebrafish, administration via skin), are poor models.

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

We will first conduct cell and molecular studies confirming our approach; organoid and other preanimal studies were considered but not deemed suitable, as we are depending on systemic effects - our molecule localises in the blood, and we will monitor effect by: a. changes in locomotion or b. changes in nerve transfer from brain to leg or c. changes in respiratory rates - all requiring whole animal models.

In cell studies, we will check for any potential toxicity measuring cell viability and cell death. Where possible we will use human kidney and human liver cell cultures because compounds accumulate in the kidney and liver for metabolism and clearance. We will also test electric changes of cell membranes to confirm cardiac safety.

Before proceeding to in vivo efficacy studies, we will check in vitro, using specific strain of bacteria, whether the compound could cause mutations in DNA, which would be undesirable in humans. If our reversal agent proves to have an acceptable toxicity profile in each of these tests, we will move to in vivo studies. It is important to confirm if the compound is safe to use in animals and does not introduce any long-term side effects, so in the future it could be applied in the clinical settings. We considered using a computational approach, and various predictive models, but our compound is new chemical matter, follows a very different mode of action and has physical properties divergent to those the models are based on, so they cannot provide accurate predictions of our system.

Further to this, we are following this research with the aim of testing our therapeutic compound in humans, and so need to generate preliminary in vivo animal data to underpin further development and eventual application.

To ensure we do a thorough search for alternatives we have studied FRAME advice https://frame.org.uk/resources/searching-for-alternatives/

We have reviewed all relevant datasets available in EURL-ECVAM collection

https://data.jrc.ec.europa.eu/collection/id-0088 but proposed alternatives were not suitable. Our reversal compound has different properties to typical pharmaceuticals or small molecules which the datasets included, so we cannot use those alternatives to predict the properties of our compound.

We have also searched literature, and as the compound is novel we will not be duplicating already published work. However we will design our studies in a similar way to those published on different compounds to be able to compare efficiency of the reversal compound.

Why were they not suitable?

Use of organoid and other pre-animal studies is not possible in this project as we depend on complex behavioural changes and physiological systems acting together, which cannot yet be mimicked without animal models. For our drugs of abuse research, and for our NMBA work, we need to demonstrate that our molecule can act across biological barriers - our molecule is localised in the bloodstream, but must remove NMBA agents from the neuromuscular junction. We cannot yet mimic this complex process of passing between biological barriers in simpler models. Computational prediction is not suitable for our molecule, as its structure and mode of action are very unusual, so not well fitted to the databases used to construct computational 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 have used the minimal number of animals that will ensure we generate robust data, considering the number and types of studies we are likely to perform over the licence period. We will use two different neuromuscular blockade drugs, and have two control groups - one without treatment, and one with reversal using the current state of the art, to ensure we can draw valid conclusions. We will use up to 300 rats in our neuromuscular blockade reversal studies, which will allow us to generate reliable data that will provide a firm readout on whether our technique works in vivo, reducing the risk of having to repeat experiments. We will use groups of up to 10 rats to study several neuromuscular blockade drugs, along with controls, and best in class FDA approved drugs and published molecules as reversal agents, along with the Reversal agents. We anticipate testing 4-6 neuromuscular blockade drugs, with 2 control competitor reversal agents, and our Reversal agents needing 40 rats per neuromuscular blockade drug. We add an allowance for technique development and variability, for a total of up to 300 rats in total.

Likewise, with mice, the minimum number of animals will be used to provide interpretable data on movement and drug of abuse reversal. We will use up to 140 mice in our acute maximum tolerated dose study, to allow us to test 6 different doses in the 2-200 mg kg-1 range for each of the Reversal agents, and a control group, with up to 10 mice in each group. We will use similar groups to study chronic administration, looking at long term toxicity, and so a further 140 mice. We have added an allowance for technique development and variability, for 360 mice in total.

Up to 1500 mice will be used to study drugs of abuse, allowing us to test up to 20 drugs of abuse with up to 10 mice in each group, with each of the Reversal agents to generate reliable data, along with control groups and in comparison with reversal drugs used currently. We will use various experimental designs to generate the most reliable data, using pilot groups for each drug of abuse.

We will also be able to test overdose reversal (up to 5 drugs of abuse, up to 8 mice per group, one control group, and the Reversal agent groups, up to two commercial/comparator state-of-the-art groups), with an allowance for technique development, so testing up to 300 mice.

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

We have minimised the number of control groups and Neuromuscular blockade drugs to be tested to the fewest needed to gain proof-of-concept. We have done likewise in our drugs of abuse study. This has allowed us to minimise the number of animals used in this study. We have also designed the study so that initial data will be generated in non-animal studies, including biophysical characterisation and cellular toxicity assays, minimising the number of animals needed. Our experimental design takes into consideration the NC3R guidelines. For most of the quantitative experiments, design will be based on PREPARE guidelines and sample sizes may be set using power analysis. Otherwise, we will use the minimum number of animals to provide an adequate description, generally based on previous experience (our own or from the literature). We will also use the same animals to collect information on distribution by sampling blood and urine, which will also allow us to gather information on clearance and metabolism, maximising information gained, and minimising 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 the number of animals used in this project we will purchase rats for each procedure, and not breed them ourselves, to maximise the efficiency of the process. Mice used in the project will be obtained from central breeding facility and we do not anticipate creating surplus breeding of those. Optimal neuromuscular blockade drugs will be chosen by identifying the drugs which are trapped sufficiently strongly (in buffer solutions, by the Reversal agents) to be effective in the blood in vitro before commencing any animal studies to ensure the maximum chances of successful reversal in vivo.

Wherever possible we will share animal tissues with other scientific groups to minimise the 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.

We will first conduct a maximum tolerated dose study on mice to confirm the safe dosing window for subsequent studies. We will use a gradual escalation procedure, where the lowest doses are tested first, allowing us to terminate the experiment as soon as moderate side effects occur. This will minimise the number of animals exposed to any side effects.

The rats for our NMBA study will be fully anaesthetised throughout the experiment where they will be administered NMBA and our reversal agent. Rats will be ventilated to allow adequate oxygenation whilst under NMBA. Rats will be killed before regaining consciousness. Those steps will minimise pain, suffering and distress. Rats are well characterised in the study of NMBAs, with many features applicable to human biology, with their larger size facilitating surgical procedures, their more similar nervous systems and more similar response to toxic compounds. As such, rats are ideal for this section of the study.

Doses for the drugs of abuse study will be chosen to minimise distress, by using the least possible dose that causes an observable effect, based on previous studies, and so minimise the impact on the mice. Mouse locomotion activity will be monitored in e.g. behavioural arenas or using in-cage monitoring such as digitally ventilated cages (DVCs) during the experiment to confirm effects of drug of abuse and then effectiveness of the Reversal agents. Animals will be closely monitored for unexpected adverse effects of substances and humanely killed if those are noted.

Similarly, to NMBA experiments, during an overdose of drugs of abuse experiments mice will be under anaesthesia throughout the procedures and will be humanely killed before regaining consciousness.

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

In order to be able to confirm effectiveness of our compound in reversal of substances used in the clinic we need a mammalian model to recapitulate processes relevant to human use. Rats are a preferred species that can be used for neuromuscular blockade drugs, with similar electrophysiology to humans, and of sufficient size to attach electrodes to nerves to directly measure recovery from neuromuscular blockade drugs with minimal suffering.

The drugs of abuse study depends on studying behavioural changes (rates of movement) before and after administering a reversal agent. As such, mice are the least sentient species it is possible to use to the best of our knowledge.

To be able to take the Reversal agents towards the clinic, we must first confirm that its mode of action is effective in animal models, and so these experiments are essential.

We are unable to perform the same studies on less sentient animals e.g. fish due to different physiology, administration routes and outputs measured (e.g. muscle tone or breathing rates). Similarly, we would not be able to perform the same experiments on neonates.

Two of our experiments are designed to be done under terminal anaesthesia to minimise any distress or suffering.

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

Administration of the NMBA drugs and reversal agents and experiments with overdose of drugs of abuse will be conducted fully under terminal anaesthesia and animals will be killed before they regain consciousness.

Any animals going through surgical implantation of cannula will be subjected to standard peri-operative care and will be allowed to recover fully before any further procedures.

Where behavioural testing is performed, animals will be acclimatised to the set up before the experiments commence to minimise any distress.

Mice displaying any signs of adverse response will have increased inspections and more regular weight checks progressing to daily if/when necessary.

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

ARRIVE guidelines will be followed. The PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines will be followed for planning experiments. The procedures with care guidelines (from the Research Animal Training resources database) and dosing guidelines (Morton et al.) will also be followed. This will be continuously updated and complemented with the relevant literature in refining procedures.

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

We will stay informed by constantly reviewing the literature, getting information form the NC3Rs webpage, the NORECOPA (Norway's National Consensus Platform for the advancement of the 3Rs) and the local NIO newsletter. We will also benefit from the depth of experience and wide expertise of the animal facility, who will raise any potential advances.

55. Impacts of Environmental Factors and Pharamaceutical Exposures on Reproductive Health

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

Reproduction and fertility, Fertility preservation, Cancer treatment, Testis, Ovary

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 primary aim of the project is to understand the effects of exposure to environmental factors (e.g. chemicals) and medicines (e.g. cancer treatments) on the development and function of the male and female reproductive system, including impact on future fertility.

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 recent decades there has been a dramatic decline in human fertility, and this is considered to be largely due to external factors. The development and function of the male and female reproductive systems are sensitive to environmental chemicals and pharmaceutical (medicines) exposures. However, the effects of many exposures remain to

be determined and the way in which they can affect reproductive development and fertility are not clear. Understanding how these exposures affect reproduction and fertility will help us to develop strategies for prevention and treatment to protect and preserve reproductive function and fertility in humans, including those undergoing cancer treatment(s) that impair future fertility.

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

The project will result in new understanding of the effects of environmental and pharmaceutical exposures on reproduction and fertility, which we will continue to publish in leading journals in the field. Results will be presented at national and international conferences for reproduction, fertility, endocrinology and cancer. The project will also inform development of patient materials relating to the effects of cancer treatment and environmental impacts on fertility. Novel treatments to preserve fertility will be tested during the project and if successful will lead to clinical trials for new medications that can be combined with cancer treatment (and other gonadotoxic therapies) to reduce the harmful effects of these in patients.

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

The project will benefit society by providing information that can be used for further education about reproductive health. Furthermore, this data can be used to modify exposure to agents that are shown to be harmful to reproductive health. This will mainly be focused on male fertility but will also include studies investigating effects on female reproduction and other body systems in both sexes. Benefits to society will be realised in the short-term through publishing and presenting the findings, whilst in the medium- to long-term this project will result in benefit to human health through engagement with regulatory authorities to legislate on exposures and identification of novel agents that can be used to protect and preserve fertility and reproductive function.

The academic community will benefit from the research through understanding the development and function of the reproductive system and how this may be affected by exposure to environmental factors and medicines. This includes academics working in reproduction, fertility, stem cells and cancer.

Key beneficiaries of this project will be children and young adults with cancer, alongside clinicians/ healthcare providers involved in their care. One of the key aims of the project is to determine the effects of childhood cancer treatments on future fertility in males and females, and to develop strategies to preserve fertility in these patients. In the short-term, the results of the work will help clinicians discuss fertility and fertility preservation options with patients. Over the longer term, treatments to preserve fertility will be developed for clinical use, which will be of benefit to cancer patients.

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

The outputs will be maximised by making use of existing channels for dissemination and collaboration. We will involve our international consortium to perform collaborative research that will make the best use of our precious human tissue resource and technical expertise. We will publish all results, which will include successful and attempts to develop new treatments for fertility preservation. Importantly, we will also publish studies with negative findings with regards to such treatments, to avoid unnecessary replication or duplication of studies. All publications will be open access to ensure all academics and general public can access the results of the research. We will also continue to present our findings regularly at the international conferences of the leading societies and

organisations for reproduction (e.g European Society for Human Reproduction and Embryology), fertility (Society for Reproduction and Fertility, International Society for Fertility Preservation) and cancer (International Society for Paediatric Oncology). Importantly, we will continue to report on our work and summarise key findings on our social media channels and website to disseminate to a public audience.

Species and numbers of animals expected to be used

- Mice: 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.

Our work is largely focused on the impacts of exposures during fetal or prepubertal life on subsequent reproductive development and fertility. For such studies experimental models (including animal models) are required. Mice have similarities to human from the perspective of gonadal development and function, making them suitable for studies relating to fetal, prepubertal and adult stages of testicular and ovarian development. The use of rodents for in-vivo and in-vitro studies has proved predictive of effects in humans in many cases and has informed the development of our subsequent studies (in-vitro or xenograft) using human gonadal tissue. Where there are key differences between rodent and human, we have developed a xenograft model, which provides the only feasible way of dynamically studying fetal/prepubertal human testis and ovary development in an 'in-vivo' system.

In order to determine the long-term effects of exposures on fertility, offspring and/or the potential for inter-generational effects in-vivo approaches in rodents are still necessary.

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

Exposure to environmental chemical(s) or pharmaceutical(s)

Animals will be exposed (oral, injection) to environmental chemicals or pharmaceuticals (medicines). Frequency (typically once daily to once weekly) and duration (typically 1 day to 1 week) will depend on the chemical/drug being investigated with a focus on mimicking the way in which exposure occurs in humans (e.g. dose, route and frequency). Some animals will be exposed to a combination of drug(s) considered to damage the testicle/ovary and another drug aimed at preventing this damage. Animals will be monitored for up to 6 months after exposure and/or mated to assess effects of exposure on fertility, offspring and/or subsequent generations.

Substances that effect gonadal homeostasis

Animals will be exposed (oral, injection) to small molecule inhibitors, agonists/ antagonists and antibodies to alter gonadal homeostasis and/or selectively impact specific cell populations to investigate the role of specific cell populations on mediating environmental chemical or pharmaceutical effects. Volumes and frequencies of administration will be in accordance with local guidelines which are in line with or more restrictive than recommended maximum volume limits outlined in LASA good practice guidelines. Animals will be monitored for up to 6 months after exposure and/or mated to assess effects of exposure on fertility, offspring and/or subsequent generations.

Transplantation of tissue/cells

Some studies will also involve transplanting human tissue/cells under the skin or into the testicle/ovary of the animal. The primary purpose is to test the effects of substances described above on human gonads (cells or tissues). Mice may be castrated/ovariectomised to remove endogenous hormones so that hormones generated from the transplanted human tissue/cells can be measured. In some cases we will use mouse models of cancer to test the effects of substances simultaneously on the transplanted tissue/cells and tumour tissue/cells.

For transplantation under the skin, male and female animals will be castrated or ovariectomized

(testicles or ovaries removed) under general anaesthetic, a procedure which takes approximately 20mins. Castration involves making a cut (0.5cm) in the scrotum and removing the testicle before closing the wound with sutures. Ovariectomizing involves making a dorsal cut (0.5cm) and two small incisions through the peritoneal wall to access and remove the ovaries. These animals will then have up to 6 small pieces (~1-3mm3) of tissue placed under the skin (approximately 15 mins procedure time) either at the same surgery as the removal of the gonads, or alternatively several weeks following removal of gonads.

For transplantation into the testicle under general anaesthetic, a cut (0.5cm) will be made in the scrotum and each testicle will be pulled out through the cut. A cut (1-2mm) will be made in the testicle and tissue/cells will be placed just under the surface of the testicle or injected into the ducts of the testicle. The testicle will be stitched and placed back into the scrotum and the scrotal wound stitched. This procedure takes approximately 30 mins. For transplantation into the ovary under general anaesthetic, a dorsal cut (0.5cm) and two small incisions through the peritoneal wall to access the ovaries will be made, with tissue/cells injected directly into the ovarian stroma.

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

Exposure to environmental chemicals, pharmaceuticals and substances that affect gonadal homeostasis

Typically, animals being given substances by oral routes or following injection using standard routes will experience mild, transient pain and no lasting harm. Additional possible effects on the health of the animals include weight loss and infection.

Transplantation of tissue/cells

Animals having minor surgery to implant tissue under the skin or into the testicle/ovary are expected to recover quickly and will be given painkillers and post-operative care just like people recovering in hospital. Animals having testicles/ovaries removed, or tissue/cells transplanted into the testicles/ovaries, are expected to experience acute pain and this will be treated with painkillers. Deaths arising from anaesthesia or surgical complications are not expected (<1%)

If any of these procedures result in or induce evidence of suffering in an animal that is greater than the anticipated severity and/or duration the animal 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 [such as providing wet mash, additional warmth or topical treatments].

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)?

Exposure to environmental chemical or pharmaceutical - 80% mild, 20% moderate

Exposure to substances that alter gonadal homeostasis – 80% mild, 20% moderate

Castration - 100% moderate

Transplantation of tissue/cells under the skin - 80% mild, 20% moderate

Transplantation of cells/tissues into the testicle/ovary - 100% moderate

Mouse cancer models - 60% mild, 40% moderate

Breeding and maintenance mice 10% mild and 90% sub-threshold

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 have developed experiments to investigate short-term environmental and pharmaceutical exposures using human and mouse tissues and these will be used to replace the need for animals for some aspects of the project. However, results of studies involving tissues in a culture dish cannot be used to mimic the way in which gonadal tissue is exposed to chemicals (primarily through the circulation), or the impact of systemic factors (e.g. hormones, growth factors) in mediating the effects of exposure. In-vitro models cannot be maintained long enough to study pathways of regeneration/repair. Furthermore, in-vitro models cannot be used to study later/adult disorders or effects on offspring and are also considered non-definitive by clinical and regulatory bodies. Lastly, we cannot conduct these studies directly in humans other than in a descriptive/observational way.

Experiments on animals provides the opportunity to perform in-vivo studies, whilst implanting the human tissue to animals offers the potential to experiment on human testis and ovary during normal, dynamic development over prolonged periods (weeks/months). This transplant model provides humanrelevant results that may directly inform regulators and clinicians.

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

Epidemiological and experimental data

We have performed (and published) systematic reviews for existing epidemiological and experimental data relating to several of our planned exposures. We will continue to review

(and publish) the epidemiological and experimental (animal and human) evidence for the reproductive effects of each drug prior to commencing new experiments.

In-vitro studies (mouse/human tissue/cells)

We have developed experiments to investigate short-term exposures using mouse/human tissues/cells and these will be used to replace the need for animals for some aspects of the project.

Tissue resources

We will aim to identify existing tissues available from other studies, including our own tissue archive, that might be used to determine the gonadal effects of exposure. We will also work together with our existing collaborations/consortia (e.g. ORCHID-NET) to co-ordinate experiments and maximise use of existing tissue resources.

Why were they not suitable?

Epidemiological and experimental data

Our previous studies have highlighted the lack of high-quality follow-up data on patients and failure to provide definitive answers on the effects of exposure on fertility, hence the need for the proposed studies involving direct exposures. We will continue to review (and publish) the epidemiological and experimental (animal and human) evidence for the reproductive effects of each drug prior to commencing new experiments.

In-vitro studies (human tissue)

Results of studies involving tissues in a culture dish cannot be used to mimic the way in which gonadal tissue is exposed to chemicals (primarily through the circulation), or the impact of systemic factors (e.g. hormones, growth factors) in mediating the effects of exposure. In- vitro models cannot be used to study long-term regeneration/repair, effects in adulthood or on offspring and are also considered nondefinitive by clinical and regulatory bodies.

Tissue resources

Despite our work within large collaborative networks, there remains a relative lack of existing material from other studies that could be used to replace animals in the present study. However, should additional material become available through existing and new collaborations, these will be used to replace new animal studies.

Only when the necessary information relating to the cause or mechanism for gonadal effects cannot be determined from alternative models or existing data will animal studies be conducted.

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 extensive experience and results from previous studies using a similar approach guides our choice of animal model, number of animals required and experimental design. This ensures that the minimum numbers of animals are used, whilst ensuring conclusive results without wasting animals or needing to repeat experiments. We also have a statistician as part of the team who advises on experimental design and assists us in calculating the number of animals required based on the results of our own earlier experiments. This allows us to calculate the minimum numbers of animals required to draw definite conclusions of an 'effect' or 'no effect'. Our calculations typically show that we need groups of 4-8 animals to achieve this. The estimated total number of animals required for this application are based on previous usage for similar experiments and the anticipated number of experiments for the duration of this licence.

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

We have designed our experiments to reduce the number of animals required by adopting a step-wise experimental approach using Experimental Design Assistant software, including random allocation to treatment groups and blinding (for treatment and analysis). For exposure studies, experimental design involves initial use of in-vitro cultures (mouse and human tissue). Our in-vitro approach using human tissues reduces the need for animals, whilst in-vitro studies using rodent tissue are more efficient than in-vivo studies as tissue from each animal can yield large numbers of samples including biological and technical replicates in order to achieve the necessary power. These two factors will significantly reduce the number of animals required compared to in-vivo studies.

Given the inter-individual variability between human samples, studies involving transplant of human tissues into animals will be designed to minimise the impact of this variation by using a paired design for exposure. This statistical approach has been used in our laboratory for numerous previously published studies and involves a two-factor analysis (two-way ANOVA) that can account for inter- individual variation, hence reducing the number of animals required to achieve statistical significance.

Power calculations to determine the number of animals required for each experiment have been derived from results of previous studies using the same approach and have been supported by a statistician working with the team. Wherever possible, we design our studies to ensure that multiple groups (e.g. different treatments and dose responses using the same control animals) can be included in each experiment, thereby reducing the need for repeat experiments. Each experiment is designed to generate sufficient tissue for multiple analyses (e.g. protein, DNA, gene expression) and prevent repetition of experiments to perform new analyses. Multiple tissues will be obtained from each experimental animal to reduce the requirement for additional/repeat experimentation.

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

Where required, pilot studies will be performed to determine the required number of animals and for dose-finding studies. This will reduce wastage of animals and ensure that each experiment is designed to answer the specific research question.

For in-vitro experiments involving animal tissues, we will utilise post-mortem tissues from control mice that have been used for other experiments and will also use surplus stock animals wherever possible.

Multiple tissues will be obtained from each experimental animal to reduce the requirement for additional/repeat experimentation and surplus material will be fixed of frozen for future analysis or cryopreserved for use in future in-vitro experiments.

We will also work together with our existing collaborations/consortia to co-ordinate experiments and maximise use of existing tissue resources and reduce the number 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.

The project will use animals for in-vivo and in-vitro experiments. We have developed in-vitro approaches that involve culture of gonadal tissue to determine the direct effects of exposure using tissue obtained from animals post-mortem reducing pain, suffering and lasting harm the animals that can occur with in-vivo experiments. For short-term exposure experiments, in-vitro models (mild severity) will be used to reduce the severity experienced by the mice, whilst transplantation approaches (moderate severity) will be reserved for experiments involving long-term monitoring postexposure or requirement for a more physiological exposure. Experiments are already designed to ensure that the end-points are as early as possible whilst ensuring the research question can be answered.

Some aspects of development, for example of fetal germ cells, differ in rodents and humans. Also, long- term development of the tissue cannot be achieved in an in-vitro model and can only be achieved using transplantation into a host animal. This demonstrates the need for transplantation using human tissues. The latter also enables us to compare function, regulation and susceptibility of rodent and human fetal gonad, which is invaluable in experimental planning and design/choice of appropriate model. The transplantation procedure is performed under general anaesthesia to reduce pain and distress and analgesia is administered to reduce pain following the procedure. The procedure has been successfully used by the team for >15 years and complications of surgery are rare.

Animals will receive all standard husbandry care and no standard treatments will be withheld. Animals will be closely monitored for any unexpected problems or distress throughout the experiments to ensure that they remain in optimal physiological state. If animals are found to be suffering unexpectedly, action will be taken immediately, veterinary advice will be sought and if the problem cannot be quickly resolved they will be humanely killed, ensuring that animals do not experience lasting harm and that the intensity and duration of any adverse effects is minimised.

We will continue to review our surgical approach and experimental design and, where possible, refine our methods to minimize harm to the animals.

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

Non-mammalian animals are not suitable for this project because gonadal development and function is significantly different compared to mammals. Therefore, the results of such experiments would not be relevant to humans. Gonadal development and function in animals used in this project is known to be similar to human and therefore the results of these studies will be relevant to the development of human studies e.g. clinical trials.

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

Refinement of the techniques to minimise potential suffering for the animals will involve regular monitoring of animals for pain following surgery and the type of painkillers given or the duration for which they are given will be refined if required. Additional measures such as soft bedding or supplementary heat will be used to help keep animals comfortable after surgery. Animals whose immune systems do not fully function will be kept in clean sterile conditions to avoid infection. For exposure studies involving new chemicals/medicines, careful ‘dose-finding’ studies informed by published literature will be performed in small numbers of animals, which will minimise potential for toxicity in future studies. Animals will be monitored for signs of ill health such as weight loss, skin irritation, changes in colour, altered mobility, lumps, breathing problems or diarrhoea. If these are observed, animals will be treated accordingly, and animals that develop severe effects will be humanely killed. Should this occur unexpectedly, procedures involved will be reviewed and refined as required.

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

We will continue to design our research according to the PREPARE framework. We will also follow guidance from NC3Rs to ensure that we are making the most efficient use of animals, minimising harms and seeking replacement models where possible. We will continue to regularly review the literature to identify new approaches to gonadal development and function that can be used to replace (e.g. computer modelling), reduce (e.g. organoids) or refine (e.g. microfluidics) studies involving animals.

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

We will keep up to date with NC3R information through visiting the website, reading the NC3Rs blog and via the NC3Rs newsletter, to which we are subscribed. We will attend the regular updates on animal welfare and NC3R updates provided locally by the animal facility veterinarians. We will also adopt any new guidance from NC3R as and when it becomes available.

56. Understanding and treating a rare neurodevelopmental disorder

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

Epilepsy, Neurodevelopment, Neurodegeneration, Genetically altered, Mouse

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?

We want to understand how mutations in a gene that is responsible for making all other proteins in nerve cells and muscle cause severe early onset epilepsy, intellectual disability and severe developmental delay, and we want to find and test new treatments for this condition.

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 is important because the disorder that results directly from mutations in this gene is a devastating childhood condition for which no treatments exist other than (often ineffective) medication to control seizures. In addition, the gene we are studying is closely involved in learning and memory and the processes that lead to neurodegeneration as well as disorders of the heart like cardiomyopathy.

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

We now have a range of mouse models of neurodevelopmental disorders caused by mutations in eEF1A2 and an understanding of how the different mutations work. We know how to assess the effects of the mutations in the mice in a way that reflects what is seen in patients (using EEG, for example) but aim to find ways of measuring abnormal acitivity in the brain in more refined, observational, ways. By the end of this project we hope to have tested a range of possible therapies, both drugs (novel and repurposed) and nucleic acid-based. All of these results will be written up as open access publications and publicised through our website and other social media, in consultation with the local communications team/press office.

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

The current beneficiaries of our work are the academic community, particularly medical researchers, who have access to novel, well characterised lines of mice modelling clinically important and other mutations in eEF1A2. The academic community will also benefit from the publications that result from our work and the mechanistic insights we obtain.

The ultimate beneficiaries will be patients and their families affected by neurodevelopmental disorders resulting from mutations in eEF1A2. We are in a strong position to engage with this group because I set up and maintain a website largely aimed at the families of newly diagnosed children. We recently carried out a survey of eEF1A2 families to ask about their priorities for research, and the responses showed an even split between parents wanting gene therapy and those preferring other forms of medication.

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

The mouse lines will be invaluable for any groups wanting to test therapies, whether genetic or drug based. In the past we distributed our antibodies to well over 50 separate labs on a no-strings basis, so we are confident that we can continue to engage with colleagues and collaborators on an active basis. The results will be disseminated through the normal channels of presentations at scientific conferences and publications both as preprints and then in the peer-reviewed literature. Data will be deposited in appropriate open access databases. The Establishment Press Office has extensive experience of generating press interest in publications and we will collaborate with them for any papers published.

Species and numbers of animals expected to be used

- Mice: 6500

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 because although the gene we work on is active in cells grown in the lab, it is impossible to study because these cells also have another form of the same gene that swamps any effects of changing the gene we need to study. We also need to look at the effects in a whole animal because the human disorders we are modelling exert their main effects on seizure susceptibility, behaviour, strength and learning ability, all of which are hard to model adequately in replacement systems. We need to look at animals from soon after birth until old age because the human disorders onset soon after birth but can have changing effects on the body through to adulthood.

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

Animals will typically be born with a specific genetic defect that has a minimal effect on behaviour and lifespan when present in one copy but which, when present in two copies, result in neurological problems. In these cases the mice will be killed by schedule 1 killing while still moderately affected (failure to gain weight, more restricted patterns of movement). The mice with one copy of the genetic defect will usually be analysed by observation and weighing followed by a variety of motor tasks and behavioural assessment (not involving pain or food deprivation). In some cases, the mice will have a small wireless chip injected, of the sort used to tag dogs, in order to detect their movements in a cage of group housed animals. Some animals will have drug treatments administered; these will be of well characterised drugs that we have predicted from lab experiments to be likely to reverse some of the changes associated with the specific genetic mutations we are studying (dosage and timing will be established once these drugs have been identified). Other groups of animals will have injections of a small synthetic piece of DNA (called an ASO) into the brain under general anaesthesia when they are neonates and will subsequently have their behaviour monitored.

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

The main source of adverse effects will be in the homozygous mutant animals (i.e. those with two copies of the abnormal gene). They are likely to fail to gain weight after weaning and to move less freely around the cage but will not be kept any longer than 23 days or after 20% loss of body weight so these adverse effects will be transient. There is no evidence from human studies that these conditions are associated with pain. Treatment with drugs or ASOs may be associated with mild, transient adverse effects but these will have to be established through pilot studies.

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 be mildly affected. A small proportion, probably <20%, will be moderate.

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?

In all experiments concerning the gene we work on, eEF1A2, we have to study animals rather than cultured cells. This is because the switch from eEF1A1 to eEF1A2 occurs in terminally differentiated cells, that is, cells that have irreversibly turned into muscle or nerve cells. In every cultured cell line (i.e. cells that can be grown in the lab) we have examined, the cells express eEF1A1 at high levels (10-100 fold higher than eEF1A2 even in highly differentiated neuronal cell lines). This means it is very difficult to detect any effects of changes in eEF1A2 as they are obscured by the compensating presence of eEF1A1. We use biochemical techniques where possible to understand the consequences of the mutations that cause epilepsy and intellectual disability, but any effects on memory and behaviour can't adequately be studied without an animal model. For the ASO work (small synthetic stretches of DNA that can be used to block gene expression), we test candidate molecules for efficacy and specificity in transfected cells in culture, and then in primary neurons, but ultimately have to test in animals to see if we can change the phenotype in terms of seizures and behaviour.

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

Cell lines and zebrafish embryos.

Why were they not suitable?

Cell lines were not suitable for the reasons stated above. We removed eEF1A2 from zebrafish but this has no apparent effect as zebrafish have three other genes that compensate. This meant we could not get useful information from zebrafish embryos.

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 estimate is based on numbers used in similar, closely related projects over the course of my previous PPL. Power calculations will be carried out for specific tests/lines after pilot studies are completed.

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

We will use online tools like the EDA for drug treatments, and seek advice from local statisticians on an ongoing basis.

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 for each new line and each test. We keep breeding lines to a minimum and aim to use all animals where possible (including using heterozygotes which have had minimal nonprocedural phenotyping for breeding purposes). We make a tissue archive for each new genotype which can be used within our group or shared with others.

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 which have been genetically manipulated using a gene editing technique that is highly efficient. There is no valid alternative to any of the techniques we are using, all of which have been chosen to reflect the human conditions we study but with as little stress as possible to the animals. We will consider the most refined administration routes for each potential treatment that is compatible with the substance being administered and will use anaesthesia and analgesia where required. We are actively working to find less invasive ways of detecting seizure-related behaviours in our lines, to minimise the use of EEGs (which involve transfer to another PPL). We use both in-cage monitoring and MoSeq (Movement Sequence) analysis of video recordings (we do not yet know if differences detected by MoSeq coincide with seizure activity but are actively working on this).

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

The gene we study is not switched on properly until well after birth and we can’t use anaesthesia for behavioural and observational testing. Less sentient species do not have the gene, which is restricted to vertebrates.

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

Animals with two copies of the mutant gene will be monitored daily from weaning and will be humanely killed before their condition deteriorates beyond moderate severity. Animals doing behavioural tests will be habituated to handling and put through a standard pipeline for tests, many of which will be carried out using the same equipment (maze, open field) adapted for multiple purposes. Animals receiving treatment will be monitored regularly following treatment and treated homozygotes will be monitored daily from weaning as above.

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

We will follow the PREPARE guidelines from Norecopa and be mindful of the ARRIVE guidelines for reporting, since an awareness of ARRIVE also helps with experimental design.

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

I receive the NC3Rs newsletter so am well informed of advances. Via my role on our local AWERB I receive and read regular updates from the ASC and Norecopa, for example, together with The Hub AWERB newsletter. I ensure that I pass on new developments to people in my lab (we started using tube handling as soon as we heard about it, for example).

57. Genes influencing calcium, bone, endocrine and neurodevelopmental disorders and their treatments

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

Calcium Homeostasis, Endocrine Tumours, Bone, Neurodevelopment, Therapy

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 aims of this project are to improve understanding of the causes of disorders of calcium, bone, endocrine metabolism and neurodevelopment (linked to two specific genes), that may involve the development of endocrine tumours involving different organs, osteoporosis, rickets, kidney stones, cataracts, diabetes, seizures (epilepsy) and autism. An improved understanding of the underlying biology of these disorders will help researchers to identify therapeutic targets and develop new drugs, which is currently hampered by our lack of knowledge of the abnormal physiological processes associated with these 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?

Our research studies focus on identifying the genetic causes of complex disorders of calcium balance, bone metabolism, kidney failure, tumours of endocrine glands (organs or glands that secrete hormones directly into the blood), e.g. pituitary, parathyroid, pancreas, adrenal, ovaries, and testes, and neurodevelopment. Our ultimate goals are to improve diagnosis and treatments for patients with these diseases. To achieve these goals, we need to study the abnormal physiological consequences of the genetic mutations identified in patients, and the only feasible way to accomplish this is to generate mutant mouse models for these diseases.

Circulating levels of calcium in the blood are tightly regulated through the actions of the parathyroid glands, bone, liver, gut and liver. When blood calcium levels fall, the parathyroid glands secrete parathyroid hormone (PTH), which acts on bone to increase calcium resorption, the kidneys to increase calcium reabsorption and to increase levels of active vitamin D which in turn acts on the gut to increase calcium absorption. All these actions result in an increase in blood calcium levels which is detected in the parathyroid glands leading to a decrease in PTH secretion.

The disorders of calcium balance, which may be present as increased or decreased levels of calcium in the blood or urine compared to normal, result in many symptoms, including kidney stones, abdominal pain, pathogenic deposits of calcium salts in soft tissues, psychiatric disturbances, excessive urine production, cataracts, involuntary muscle contractions, and osteoporosis, as well as other disorders such as cardiovascular disease and diabetes. Moreover, calcium is an essential nutrient during pregnancy and lactation (production of breast milk) contributing to bone development in the foetus and neonate. As a result some women may lose bone density during pregnancy and/or lactation. Improved understanding of how disorders of calcium balance affect pregnancy and lactation is therefore a goal of our studies.

Calcium disturbances may have multiple causes including: single gene disorders of calcium balance or bone metabolism; endocrine (parathyroid gland) tumours, or tumours of other organs (e.g. lungs and kidneys) producing PTH related compounds; kidney failure; or decreased PTH secretion which can lead to lower levels of calcium in the blood. Many of these disorders are relatively common and place a heavy burden on health care systems. For example: osteoporosis characterised by low bone mass affects ~3 million people in the UK and leads to increased risk of fractures; kidney stones which can be caused by elevated levels of calcium in the urine affect ~8% of the population by the age of 60 and frequently reoccur; parathyroid gland tumours have an annual incidence of 1-3 per 1,000 individuals; and changes in the development of embryonic tissue that give rise to structures within the head and neck, from which the parathyroid glands usually develop, and which may lead to hypoparathyroidism due to insufficient PTH production that may lead to lower circulating calcium levels and the development of tetany or seizures, have an incidence of 1 per 4,000 live births. There is an unmet clinical need for many of these disorders, either due to lack of current treatments, or only partially effective treatments. For example, the only current interventions for kidney stones are dietary, and there are no effective drugs, resulting in ~10% recurring each year. Parathyroid and pituitary tumours, or other cancers may be surgically removed; however, this is not always successful due to incomplete removal of the tumour and/or malignant growths away from the primary site, whilst conventional chemo- and radio-therapies are often ineffective for endocrine tumours due to their low growth rates. Current drug treatments for decreased parathyroid hormone production include calcium and vitamin D; however, these are associated with adverse effects including high calcium levels in the urine and kidney stones.

Disorders of neurodevelopment can lead to intellectual disability, epileptic seizures and behavioural abnormalities including autism, attention deficit hyperactivity disorders, aggressivity and tantrums, the causes of which are often poorly understood. The particular neurodevelopmental abnormalities that we are interested in studying, which are limited to changes in two different genes, can occur in combination with bone or endocrine disorders, or may develop in isolation depending on the genetic aetiology.

Our studies, which are a continuation of a body of work involving animal models for >20 years, will help to better understand the genetic causes, biochemical interactions, including hormone signalling, between different organs, in diseases where there are underlying disturbances of calcium homeostasis, bone metabolism or neurodevelopment. Previously, we have developed genetically modified mouse models and used these to gain mechanistic insights into the cause of diseases affecting the parathyroid, kidney and bone, as well as successfully ameliorating symptoms through the testing of therapeutic drugs. We propose to use genetically modified mouse models for disorders of calcium homeostasis and neurodevelopment as mice maintain a high enough degree of similarity to humans in the regulation of calcium. There are well developed techniques for modifying mouse genes, breeding the mice and characterising the physical appearance and biochemical characteristics that arise due to these genetic modifications and their interaction with the environment. By using mouse models, we will also be able to assess new and existing drugs, and dietary alterations, as well as providing new model systems in which novel therapies can be tested prior to their use in man. ***Thus, our studies are likely to increase knowledge of the disordered physiological processes associated with these diseases, as well as provide benefit to patients by improvements in diagnosis and treatments.***

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

We anticipate that the outputs will include new information, mouse models, publications and new treatments for patients as follows.

Full examination of the physical characteristics regulating calcium in blood and bone as well as noncalcium characteristics in novel mouse lines will be performed, which will increase understanding of disease causes (pathophysiology), and testing of therapies will increase knowledge of drug actions (pharmacology). Phenotyping of new pituitary tumour models will help improve understanding of tumourigenic pathways that will help to lead to the development of new treatments for patients. Preclinical testing of long-acting somatostatin (a peptide hormone that regulates a variety of bodily functions by hindering release of hormones) analogue compounds in endocrine tumour bearing mice to assess efficacy on reducing hormone secretion and proliferation, and increasing specific tumour cell death in pancreatic neuroendocrine tumours and pituitary tumours may lead to new patient treatments. Studying the effect of pregnancy and lactation on bone and mineral metabolism which can be affected by disorders of calcium regulation will yield new clinical insights that may lead to improved patient management. Phenotyping of abnormal neurodevelopment models may identify previously unrecognised characteristics in patients. Some of our neurodevelopmental models will involve studying mouse embryos to detect changes in expression within developing tissues that may result in defects in brain function, and identify pathways that can be targeted with drugs to correct such defects.

We are continuing our genetic sequencing studies of patients with disorders of calcium balance, bone metabolism, kidney function and endocrine gland tumours which may uncover new candidate genes and thus novel mouse lines based on mutations in these candidate genes may be generated. The biological advances from these studies will

depend on the characteristics of the novel gene candidates, as illustrated by our previous studies of such human disorders, which have revealed the involvement of factors regulating: gene expression, biochemical reactions, signalling mechanisms within cells, movement of substances into or out of cells, and hormones.

Thus, these studies will reveal new biological information that will be published in scientific journals. Furthermore, these mutant mice will provide models for testing existing and novel drugs, as illustrated by our studies of mouse models with disorders of calcium balance, and endocrine pathways, in which we have evaluated the effects of drugs that: mimic the effect of calcium or hormones, compete with the effect of calcium, and regulate gene expression.

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

In the short-term, our studies will help to understand genetic causes, cellular pathways and the interactions between different organs, in diseases underlying endocrine and metabolic disturbances of calcium, bone metabolism and neurodevelopment. For example, we will assess models of: endocrine tumourigenesis, calcium homeostasis disorders, and abnormalities in neurodevelopment. Using these models, we will be able to assess the impact of existing and new drugs and dietary alterations, as well as providing new model systems in which novel therapies can be tested prior to their use in man. These studies will also provide insights into alterations of the phenotype of genetic calcium disorders during the life-course. In particular, our research will help elucidate the effect of pregnancy and lactation on bone and mineral metabolism in wild-type and genetically altered mice. Thus, research scientists and clinicians in different disciplines from multiple fields will derive benefit from the knowledge.

In the long-term, pre-clinical studies will provide evidence for future clinical trials for researchers to test drugs that are novel, pre-existing or repurposed. Beneficiaries may therefore include patients, patients support groups, clinicians, and the pharmacology industry. We have a number of existing collaborations with pharma companies to test drugs and facilitate any promising results into clinical trials.

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

Results will be published in high impact peer-reviewed open-access journals and presented at national and international conferences. Collaborations will be established with national and international groups, and novel mouse lines will also be made available through archives, for example European Mouse Mutant Archive EMMA. We are also in regular contact with patient support groups to discuss our research findings.

Species and numbers of animals expected to be used

- Mice: 20,500

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.

We will use genetically altered mouse models, as mice provide rapid and efficient breeding whilst maintaining a high enough degree of similarity, in terms of calcium regulation and

the DNA sequence of the genes involved, to humans. The level of calcium is tightly regulated due to its roles in multiple biological processes, and disturbances of this cause many common diseases, including kidney stones, osteoporosis, many cancers including different organs, rickets, seizures (epilepsy), cataracts and diabetes. Abnormal neurodevelopment models provide tissue that can be used to study pathophysiological changes in structure and to assess changes in protein expression in organs (e.g. brain) that cannot easily be studied in humans. Mice will be studied at all life stages for phenotyping and treatment purposes and in the case of slow growing endocrine tumour models to allow time for tumours to develop. We expect to use approximately 20,500 mice over the five years duration of the licence.

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

The typical experience for a mouse maintained under this project licence will vary depending on its genotype (disease phenotype) and whether it is undergoing phenotypic analysis or experimental (e.g. drug treatment dosing or efficacy) investigation.

Example 1 - Phenotypic analysis. A typical experience for a mouse undergoing phenotypic analysis may comprise collecting blood samples for 5 days when animals will experience brief, slight discomfort and no lasting harm, followed by analysis in a metabolic cage provided with environmental enrichment for up to a week, followed by radiological imaging (e.g. by X-ray, DEXA or other scans) involving general anaesthesia to assess parameters of calcium, bone, kidney, and tumour metabolites followed by killing the animal and removal of blood where animals will only be aware of the anaesthetic administered and may briefly experience distress and no pain. Mice will typically be aged to a few months up to a maximum of 24 months.

Example 2 - Assessing effectiveness of drugs for treatment. A typical experience for a mouse undergoing experimental treatment would be administration of compounds twice weekly by intraperitoneal injection for 1 month, with administration of an imaging substance to indicate proliferating cells via drinking water for the final 3 weeks. Blood samples would be taken weekly followed by culling and terminal bleed. For endocrine tumour models the mice will be aged for months to allow tumours to develop. Typically mice will be aged to 6-12 months but on rare occasions some tumour models may be aged for up to 24 months.

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

Adverse effects specific to mouse models related to disorders of calcium homeostasis, bone metabolism, renal failure, endocrine tumours and neurodevelopment include:

Polyuria (excessive passage of urine), polydipsia (excessive thirst), abdominal pain, muscle weakness, fatigue and cardiac arrhythmias (irregular heartbeat) due to hypercalcaemia (~1-5%). Animals with piloerect coat, hunched appearance or evidence of dehydration observed by skin tenting showing no improvement within a working day will be killed.

Tetany (involuntary muscle contractions), muscle cramps, and seizures due to hypocalcaemia (<1%). Some mice that model the neurodevelopmental delay may rarely experience seizures that are unrelated to calcium levels. Animals in which tremors impinge on the ability to feed/grip food pellets will be killed.

Skeletal abnormalities due to disorders of bone metabolism (<1%) - any animals in which these impinge on the ability to consume solid food will be killed.

Disturbances in balance and/or walking in a circular pattern due to an advanced pituitary tumour (<0.5% as they usually develop after >12 months of age. Animals in which such behaviour results in physical harm or impinges on the ability to feed will be killed.

Reduced mobility and/or feeding due to tumour effects (<2%).

Increased urination or drinking due to disturbances in glucose metabolism (~10%). Animals that show evidence of dehydration due to skin tenting that does not resolve within 24 hours will be killed.

Weight loss and loss of condition due to metabolic disturbances (<5%). Animals that reach 15% weight loss that do not resolve within 48 hours 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)?

The majority of mice under this licence will be under the breeding & maintenance protocols in which adverse effects will be mild or subthreshold for approximately 80% of mice and moderate for 20% of mice. A subset (approximately 30%) of the mice will undergo the phenotyping or testing of agents (drugs) protocols in which the development of clinical signs is required and they will have moderate adverse 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?

Regulation of plasma calcium levels requires the interaction of the parathyroid glands, bone, gut and kidneys, via circulating hormones and vitamin D. In addition, calcium has different effects on different organs such as the pancreas. As this represents a whole body system, it is not currently possible to investigate and understand disease development in isolated cells, and instead whole live animals are required. In addition, drugs need to be tested in whole animals so that the responses of all the different organs can be studied simultaneously, thereby revealing on-target (i.e. efficacy) and off-target (i.e. side or adverse consequence) effects. To fully understand abnormalities of neurodevelopment, assessment such as histological examination needs to be performed in whole organs (e.g. brain) and animals. Obtaining human brain tissue samples from the relevant patients is not feasible. Analysis of altered protein expression using murine tissue is valuable for understanding aetiology and assessing potential therapies.

All the studies that will be performed will generate novel data as the questions asked cannot be answered from previous existing study data.

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

We endevour to search the literature and databases for non-animal alternatives which include the

Norway National Consensus Platform for the advancement of the 3Rs (https://norecopa.no/alternatives/alternatives-to-animal-research-and-testing/), the Non Animal

Technologies Database (https://www.nat-database.org/), the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) (https://joint-research-centre.ec.europa.eu/eu-referencelaboratory-alternatives-animal-testing-eurl-ecvam/biomedical-research_en), and the Fund for the Replacement of Animals in Medical Experiments (https://www.frame.org.uk).

In addition, we are striving to develop cell models that may be able to replace some mice in some preliminary studies. For example, we are trying to immortalise and grow cells taken from mouse organs (e.g. pancreatic islets) in the laboratory, and these could be used to test whether new drugs might be effective, before they are tested in live mice. Primary pancreatic neuroendocrine tumour obtained from patients during surgery have also been collected and digested for use in drug screening studies as a method to answer certain biological questions. In some cases, 3D organoid models can be used to test therapeutic agents prior to testing in animals. Furthermore, if new genetic variants are identified in a individuals or families that are believed to be associated with particular phenotypes, overexpression (or under-expression) studies will be performed in human and mouse cell lines to understand basic biology before any work in mice is considered.

Why were they not suitable?

While in vitro models can help answer some biological questions the regulation of calcium levels requires the interaction of multiple organs, and therefore, it is not possible to fully investigate and understand disease development in isolated cells and tissues alone, but instead whole live animals are required. For example, biochemical assessments of blood (serum or plasma) is required to identify potential changes in physiology. Organs can be harvested to assess any gross effects on architecture or structure as well as changes in protein expression of biologically relevant candidates affected.

All potential therapies will be thoroughly tested using in vitro models as much as practicable in the first instance to establish proof of concept, before employing the mouse models to address the questions of optimal dose, route of administration and efficacy, that only in vivo mouse models can 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?

The number of animals for each experiment are calculated based on effect sizes known to be of biological consequence from previous studies or the literature. Where effects size cannot be predetermined then either pilot studies will be performed using small sample sizes to establish preliminary data that can be used to estimate sample size of different groups including relevant controls. The number of animals estimated for each protocol are based on the anticipated number of experiments planned for our research, which are then combined to give the total number of mice for the project. Studies are randomised and blinded, and we take advice from statisticians regarding the numbers of animals required to ensure results are statistically significant and biologically relevant.

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 required for the experimental studies is determined by power calculations to achieve an effect size. The group is in contact with statisticians who are involved on the statistical requirements in the experimental designs. Randomisation and blinding will routinely be used. We will also employ online tools such as the NC3R's experimental design assistant for example in estimating sample sizes of groups to test various treatments for efficacy studies in the treatment of pancreatic neuroendocrine tumours. Whenever possible we will combine treatments in order to share a control group. Also, we are able to study the effects of drugs in multiple organs within an individual animal, for example in mice which develop tumours in more than one organ. Similarly, we can image the same mouse several times to study the development of organs or tumours, rather than using several mice once.

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

For breeding purposes, we will use the minimum number of mice required for each study and will plan the appropriate number of matings in advance using conventional statistical methodology. Our animal facility staff are skilled in efficient colony management. Using our experience and that of the animal technicians, we will employ efficient breeding strategies; for example, mouse lines will only be maintained whilst there is a justified use for their continued breeding. Any line with no predicted usage will be cryopreserved.

Breeding numbers are calculated taking into account average litter size for that stock as well as known neonate mortality. Breeds are set up in a controlled, time-restricted manner, to ensure that all mice born are the correct age for the study. Where breeding information is not known (i.e. for new lines) a small pilot breed will be carried out first to assess viability. These mice, if viable, can be used for the first cohort of the study, and remaining larger breeds will be set up taking into account information gathered from the pilot breed.

Genetically modified lines, which have previously been studied, will be sourced from repositories (these are carefully managed stores in which the DNA of GA mice is stored for future use), to avoid remaking of lines whenever possible. Any excess stock will be offered to other researchers to minimise wastage.

We routinely freeze multiple organs from each mouse after death for future studies. After many years of mouse research, we have a bank of frozen tissues and samples from past studies that can be employed to investigate some research questions.

In the past we have been contacted by other researchers for help with material such as blood and tissue samples that we have available, and which we have shared.

We will continue to make use of databases of human and mouse information e.g. UK Biobank and Mousephenotype.org to obtain information which will reduce the number of animals we need to 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.

We use animal models of endocrine and metabolic disorders affecting calcium homeostasis, the skeleton and kidney functions as these are often interlinked in relevant human diseases, which include: familial hypercalcaemic hypocalciuria (FHH), autosomal dominant hypocalcaemia (ADH), endocrine tumour (pituitary, parathyroid, pancreatic islet, adrenal, testes, ovaries) development, abnormalities of neurodevelopment (Marshall-Smith syndrome, developmental delay with epilepsy) and renal failure. The models we use are representative of disease in human patients and are closely monitored for signs of adverse effects when they are killed. The majority of studies will be ended before mice reach humane endpoints.

The group has established and refined methods that generate clinically relevant methods and robust information with the least suffering. We are using LASA guidelines for asepsis techniques and administration of substances.

Whenever possible for our endocrine tumour models we use conditional expression models to limit tumour development to specific organs.

For our neurodevelopment models we will employ Home Cage Activity analysis to detect phenotypic effects at an earlier time point.

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

Mice will be used in our studies, and have been chosen as they represent the lowest mammalian species which allow the necessary genetic manipulations and display sufficient similarity to human organs and physiology. Non-mammalian animals are of limited use because their method of calcium regulation differs too greatly from humans to provide relevant results. Often older mice are required in order to allow tumours to develop that are representative of endocrine tumours in humans. Some of our neurodevelopmental models will involve studying mouse embryos to detect changes in expression within developing tissues. It is possible that some biological questions can be addressed in zebrafish models in order to study very early developmental abnormalities that are conserved across species, however many studies rely upon the higher ordered brain and neural structures found in mammalian physiology. In the future it is hoped that some biological questions can be addressed using zebrafish models but at present mouse models are required.

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

We have developed expertise and experience in mouse welfare, and have refined our tests to ensure that the highest quality data is generated for the least welfare cost. For example by trying to reduce blood volumes taken whenever possible, reducing fasting periods for mice, and performing multiple imaging during a single anaesthetic.

We are keen to minimise severity and increase the welfare of these animals. To ensure this, we will use non-invasive tests that only cause temporary discomfort where possible. For administration of drugs, a small pilot study will be undertaken for new drugs, with increased cage observations and welfare checks to ensure that the drug is safe. We also aim to use long-acting drugs where possible to reduce the frequency of dosing. During every test, mice are closely observed and anaesthetics or analgesics used when appropriate.

Ageing animals will be monitored for adverse effects and any animals that reach humane end points will be killed.

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

We will consult the PREPARE guidelines and conform to the ARRIVE and OBSERVE guidelines on animal studies. We conform to LASA guidelines on administration of substances.

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

We attend termly animal welfare meetings at which an establishment representative of the 3Rs gives regular updates. We also consult the 3Rs websites for techniques and alternative models (e.g. Norway National Consensus Platform; Non Animal Technologies Database; The European Union Reference

Laboratory for Alternatives to Animal Testing (EURL ECVAM); and the Fund for the Replacement of Animals in Medical Experiments). We regular perform literature searches to keep up to date with advances in animal welfare. We also seek regular advice from colleagues who are experts in animal husbandry.

If more refined techniques become available they are discussed with the animal technicians and researchers and whenever possible implemented immediately. It would only not be implemented immediately if a study was part way through and such a change would have impacts on the reproducibility of the scientific data collected.

58. Cytokine signalling in development 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

Cytokine signalling, cancer, marfan-related syndromes, Therapy

Animal types	Life stages
Zebra fish (Danio rerio)	Embryo and egg, Neonate, Juvenile, Adult, Aged animal
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 understand how cell communication via signalling proteins called cytokines orchestrates early vertebrate development and how in addition, its deregulation results in human diseases, for example, cancer, the Marfan syndromes and Myhre syndrome.

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?

We want to understand how cells communicate with each other via signalling proteins called cytokines and how this regulates early vertebrate development and the correct development of all the organs of the body. We anticipate that this will aid in understanding the origin of human developmental abnormalities, and could thus help develop treatments. Normal embryonic development is precisely controlled by cell communication

through a number of different signalling proteins. When this signalling goes wrong in childhood or adults, either because it is perturbed by mutations that arise in key proteins that are required to transmit the signals in the cells or cells become resistant to messages telling them to turn off the signals - this can cause disease. A prime example is cancer which is a major cause of mortality in humans, accounting for about a quarter of all deaths. Out of control signalling by the signalling proteins that we are interested in leads to many different kinds of cancer and understanding how could directly result in new targeted treatments. In addition, we are working on two other rare diseases called the Marfan syndromes, which have a prevalence of approximately 1 in 5000 and Myhre syndrome that currently has unknown prevalence as the causal mutation has only recently been identified. These diseases have very few treatment options and we anticipate that our work understanding more fully their causes will give rise to new treatment options.

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

The outputs of this work include:

An understanding of the role of two signalling proteins called Activin and TGF-β in cancer. These proteins are members of the TGF-β family and are produced by different cell types in a tumour and bind to receptors on target cells. This then activates a cascade of events that result in the cell changing its behaviour. In this way they have the capacity to drive tumour development. We are pioneering the use of anti-Activin and/or anti-TGF-β therapies for different cancers, both as monotherapies and in combination with other therapies. If successful, these could be developed into a cancer therapeutic for humans in the future.

A better understanding of the role played by TGF-β family members in cancer , which could lead to new therapies, and to the development of diagnostic and prognostic markers.

One of the cell surface receptors for some of the TGF-β family members is called ACVR1. Single changes in the sequence of this receptor results in a devastating pediatric glioma called diffuse midline glioma (DMG). Working out how this aoccurs could help with the development of treatments for this fatal disease.

The TGF-β family members work by sending signals to the nucleus of target cells to induce those cells to make new sets of proteins that alter their behaviour. Mutations in the proteins that are involved in sending these signals lead to the Marfan-related syndromes (Marfan syndrome, Loeys-Dietz syndrome or Shprintzen-Goldberg syndrome). We want to understand how this occurs as this will help us develop novel therapies. We anticipate that the zebrafish disease models we generate could be used to test novel therapeutics.

One of the key proteins that is involving ed in signalling by TGF-β family members is called SMAD4. Mutations in SMAD4 lead to the rare genetic disorder called Myhre syndrome, which results in systemic organ fibrosis among other things. Understanding how this occurs could lead to novel therapies for this devastating disease. We anticipate that the zebrafish disease models we generate could be used to test novel therapeutics.

At the very beginning of embryonic development, members of the TGF-β family are involved in dictating what different cells become – a process we call cell fate specification. Our work focused on this phenomenon will be of great benefit to the basic research community and will inform tissue regeneration studies.

As part of the project we are generating transgenic lines of mice and zebrafish that make so called reporters or biosensors that allow us to visualise cell communication in vivo in real time. These lines will be of enormous benefit to the research community as they can be used in numerous other projects where a visual readout of signalling is required.

The outputs will therefore be new scientific knowledge, new animal models for use by others, publications, presentations, patents filed, tool compounds for use in research and possibly for further development towards agents that may be used in clinical trials.

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

There are likely to be multiple beneficiaries from the outputs above. These include:

The anti-Activin therapies we are developing, if successful, will be further developed in collaboration with a pharmaceutical company. Further development by a company would take of the order of 5 years, before first in human studies. The ultimate beneficiaries will be cancer patients, if we succeed in developing it as a drug.

From our other disease orientated projects, we anticipate that we might discover potential diagnostic or prognostic markers that could be developed for improved clinical management of patients. The time frame for this could be 3–5 years.

From our disease models, we may also identify possible treatments that rescue the effects of the mutations, that might ultimately be able to be developed into therapies. The time frame for this could be 5–10 years.

This work will benefit the basic research community by increasing our knowledge of embryonic development and cell biology. This will occur on the 1-5 year timescale.

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

A major mechanism to maximise the output of the work, alongside the publication of primary research papers, will be presentation of the work at big international meetings and also smaller workshops. Not only will the positive results be communicated, but where it is useful for other researchers, unsuccessful approaches will also be highlighted.

We collaborate widely, both internally and externally, nationally and internationally and this provides another avenue to share details of approaches that were ultimately sub-optimal and how methods were improved.

Where we develop new transformative methods, we will publish specific protocols papers and post on BioRxiv.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 36,000
- Mice: 8070

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 understand how cell communication through cytokine signalling as explained above is involved in orchestrating early embryonic development and how its deregulation leads to human diseases such as cancer, Marfan-related syndromes and Myhre syndrome.

For the embryonic development work we use zebrafish embryos that are less than 5 days old (before independent feeding). These embryos develop ex utero and are transparent and thus excellent for imaging and we can generate 100 embryos from one set of parents that develop synchronously. We can also study aspects of Myhre syndrome and Marfan-related syndromes in embryos less than 5 days old such as the heart development, eye development, craniofacial development, but it may be essential for the understanding of the mechanisms underlying these syndromes to study fish beyond 5 days, through juvenile stages and up to adulthood (2–4 months). This will be important to understand the effects of TGF-β pathway component mutations on bone development.

For the cancer work, we want to understand how tumours develop and spread and to test candidate therapeutics to inhibit these processes. To do this, it is necessary to work with animals that have similar organs to humans – for example, lungs, pancreas and mammary glands. This leads to the choice of mice. The main cancer types that we study occur in adults, and occasionally, young adults. Therefore, we work with juvenile and adult mice.

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

For the mouse experiments, procedures will be performed that lead to the development of cancer in mice. In some cases, this will be through injected cancer cells, for example in the mammary fat pad for breast tumours or in the tail vein for lung metastases, intrasplenic for liver metastases or injected into the pancreas to model pancreatic cancer. In other cases, mice will develop tumours as a result of a particular breeding protocol designed to mutate oncogenes or tumour suppressors in certain organs. In the breeding protocol, mice will be genotyped, most usually by ear biopsy. Sometimes the oncogenes will be inducible, and thus mice may be treated with substances to induce the oncogenes so that the tumours develop at a particular time during the mouse's life. These may be administered in the drinking water or by standard routes (intravenous, subcutaneous, intraperitoneal). We will monitor tumour development and spread when we are investigating the effect of loss (or gain) of a particular protein/proteins, e.g. a component of TGF-β family signalling pathways. This is done by palpation for mammary tumours, or by ultrasound or occasionally, MRI or micro-CT for internal tumours like those of the pancreas. Alternatively, mice may receive therapies similar to those being used or being developed to treat patients. In these cases, the mice will experience brief, slight discomfort and no lasting harm from administration of substances by injection using standard routes (intravenous, subcutaneous, intraperitoneal). Where possible, advanced imaging methods (ultrasound, micro-CT or MRI) will be used to monitor the spread of tumours and how they react to therapies. This is performed under anaesthesia. As radiotherapy is often used to treat human cancers, we will also investigate the effect of radiotherapy on tumour growth and combine it with other chemical or biological treatments. The area of interest may be marked with a fiducial marker injected into the relevant organ and radiotherapy will be carried out using an image-guided Small Animal Radiation Research Platform (SARRP). Blood may be sampled during the experiment. In some cases, in order to study metastasis, the primary tumour will be resected under general anaesthesia. Final procedures like intravital imaging will be undertaken under non-recovery anaesthesia. The mice will be aware of the administration of the anaesthetic and may briefly experience distress but no pain. At the end of the experiment, mice may also be injected with labelling

agents like BrdU, EdU or Texas-Red-tagged dextran to label proliferating cells or particular tissues (like blood vessels) respectively. Sometimes it may be necessary to fast the mice briefly or put them on a special diet that will limit auto-fluorescence prior to imaging. Extensive postmortem tissue analysis will be performed to maximise the information obtained from each animal.

For the zebrafish work, we are predominantly generating either lines expressing fluorescent reporters for different signalling pathways as explained above, or lines in which we have knocked out or mutated specific proteins in the TGF-β family pathways or other cytokine signalling pathways. These will mainly be studied at the level of embryos and larvae less than 5 days old. Sometimes reporters or transgenes may be activated by chemical agents or transient warming at 37°C for up to 1 hr. The only procedures that the adults go through are breeding and genotyping. In some cases where we are modelling human diseases like Marfan-related syndromes or Myhre syndrome, we may study the larvae up to juvenile and adult stages. This will involve observing phenotypic changes, but mainly will involve investigating the organs of the zebrafish post mortem.

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

For the majority of the zebrafish experiments, we do not expect any adverse effects as we are mainly working with transgenics that have no negative effect on development or we are monitoring the result of genetic alterations only in larvae younger than 5 days old. For a small proportion of mutants, there may be some developmental abnormalities, for example slight enlargement of the outflow tract of the heart in fish carrying genetic alterations that result in the Marfan syndromes. If we observe abnormal behaviour, such as reduced swimming activity or retreat to dark tank corners, the fish will be killed. The addition of chemicals to the water can cause transient irritation to the skin or gills. Fish displaying abnormal swimming behaviour, gill bleeding or having skin problems which do not resolve within 24 hrs will be killed.

In our project mice will develop tumours. Initially, these have little effect on the mice, but as they become larger they might affect mobility. Further, as tumours begin to spread they can affect weight, breathing, and behaviour. Depending on how much the animal is affected, the duration of the adverse effect may range from a small number of days to a small number of weeks. Additionally, the animals will experience brief discomfort from blood sampling associated with insertion of a small needle through the skin. They may also experience brief discomfort from the administration of substances or therapies by standard routes (intravenous, subcutaneous, intraperitoneal). Adverse effects may also be experienced as a result of the surgery required for tumour resection. Pain will be controlled with analgesics. The radiotherapy may cause adverse effects like weight loss, skin ulceration, reduced mobility and generalised intestinal issues, but are only expected in 1 in 500 mice using the SARRP.

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 zebrafish experiments, moderate severity is expected for about 10% of fish - the rest will be mild. Breeding is expected to be sub threshold.

For the mouse experiments, moderate severity is expected for about a third of the animals, with the rest mild. Breeding is expected to be sub threshold.

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 have been working on TGF-β superfamily signalling for almost 30 years and have gained tremendous insights into the mechanisms of signalling and the role of these signalling pathways in regulating cell behaviour from working in model tissue culture systems. However, in order to be able to investigate how these pathways regulate embryonic development, tumour formation and metastasis, and how mutations in pathway components lead to Marfan-related syndromes and Myhre syndrome, we need to perform experiments in animals for the reasons explained below.

For early development, we need to be able to study the generation of the three germ layers

(mesoderm, endoderm and ectoderm) and their subsequent patterning in a living embryo. To be able to answer our questions, we have chosen zebrafish embryos. The advantage of this system is that we can minimise the number of procedures performed on animals deemed to be sentient, by doing most of our work with zebrafish embryos and young larvae, before the onset of independent feeding. The only work that will be done on adult or juvenile fish is the fin clipping for genotyping or transient warming to induce expression of transgenes. Concerning the genotyping, as often as possible mating will be performed followed by analysis of phenotype to determine the genotype of the fish as an alternative to fin clipping. The huge benefits of the zebrafish embryos are their rapid embryonic development ex utero and optical transparency which allow a direct monitoring and visualization of development, morphology and physiology in the living organism. Recent development of novel genome editing tools (such as the CRISPR/Cas9) system has made it possible to generate mutant zebrafish lines in a highly efficient manner and the technology is easy to implement. Other advantages include ease of transient genetic manipulation, for example the generation of fluorescent reporter lines. For the Marfan Syndrome and Myhre syndrome work we are also using the zebrafish system as we can readily generate appropriate mutations in this system, and we can monitor the development of mutant embryos much more easily than in the mouse. We are also complementing this with cell-based in vitro models.

With regards to the mouse work, we only perform studies on mice that cannot be done in model tissue culture systems or in the zebrafish system. We are studying the effect of inhibiting TGF-β family signalling pathways on the development and spread of tumours. This involves multiple systems in the body (organ which harbours the primary tumour, for example the pancreas, the blood circulation, the lymphatics, the immune system). Although aspects of these systems can be modelled in vitro, we need to perform experiments in the whole animal to understand the effect of the treatments on cancer progression and to understand the underlying mechanism of action.

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

We complement our in vivo analyses with tissue culture models and organoids.

For the early developmental work, we can model some aspects of development in 3D organoids like gastruloids which are developed from human or mouse ES cells, but these organoids do not recapitulate all aspects of vertebrate development and do not develop with the spatial and temporal precision of real embryos. We are using these systems alongside our zebrafish work and they complement the animal work but cannot replace all of it.

The cell-based in vitro models for studying Marfan Syndrome (smooth muscle cells co-cultured with aortic endothelial cells) that we are generating will be very informative about the underlying mechanism of aortic aneurysms caused by the mutations in the TGF-β pathway components. We will then use the fish models as well as human samples to verify hypotheses generated in the in vitro systems.

For the mouse tumour work we are setting up an in vitro system comprising cancer cells, cancer associated fibroblasts and cytotoxic T cells to test initial hypotheses about mechanism of action of our experimental treatments before testing them in mice. This should enable us to reduce the number of mouse experiments and will make the animal experiments we perform more powerful.

I have ensured no duplication of work by publication searches through PubMed

(https://pubmed.ncbi.nlm.nih.gov/?otool=iukucllib), searching through the preprint server BioRxiv (https://www.biorxiv.org/) looking for related research. I also attend regular conferences in my fields and thus become aware of research that is not yet published.

In searching for relevant alternative methods to animal use I have used the following websites:

For immuno-oncology alternatives: https://data.jrc.ec.europa.eu/dataset/352f7dfd-05cf-434b-a96a7e270dc76573

For breast cancer alternatives: https://data.jrc.ec.europa.eu/dataset/ffebe454-ed9a-47cf-8a338cf70c1b7d38

For other alternatives: https://frame.org.uk/resources/searching-for-alternatives/ and https://www.nal.usda.gov/services/literature-searching-animal-use-alternatives

Why were they not suitable?

We can gain a certain amount of information from these in vitro systems, but there are serious limitations. With reference to the tumour work it is not currently possible to replicate the complexity of mammalian tissue structures in culture models. Moreover, the immune system only functions effectively in an organismal context with appropriate white blood cell movement and function within lymph nodes. Finally, in the process of metastasis the cancer cells move between organs. It is not possible to recreate this using in vitro models.

For our zebrafish developmental work, as stated above, the mammalian gastruloids are useful to complement the embryonic work but do not develop with the spatial and temporal precision of real embryos.

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 zebrafish work we calculate the numbers of fish required based on the numbers of mutants and transgenics that we are currently maintaining, plus those that we need to generate to be able to achieve the aims of the project. Our estimates are based on good practice in the zebrafish field and also our experience of working with zebrafish for more than 10 years. Details of how many fish we need to raise to generate mutants and transgenics are as follows.

For the maintenance of zebrafish lines, we estimate that we use 300 fish per line over 5 years.

For generation of new transgenic lines, estimates of numbers of fish needed are based on statistics on the efficiency of transgenesis from ours and other laboratories. Typically for one transgenic line we will raise 100 F0s, which will be screened by crossing them to wild type and screening their progeny for the transgene. Typically, 20-30 of the adult F0s will be founders. We will choose 2-3 of these and raise their progeny; the others will be culled. For each ‘family’ we will be raising around 60 fish which will be genotyped to identify heterozygous carriers of the transgene. The transgenic lines will be then maintained as for other wild type and mutant lines, which corresponds to about 4 tanks of around 15 fish each.

A similar strategy will be employed for the mutant fish generated by CRISPR-Cas9 technology. To produce one mutant line we raise 200-250 (‘founder fish’), which are phenotypically normal after microinjection of the CRISPR/Cas9 constructs or TALENs. To screen for germline transmission and loss-of-function alleles the F0s will be crossed to wild type fish, and their progeny will be screened as embryos for the presence of the mutation. We will pick 2-3 positive founders and raise 200-250 progeny. The others will be culled. The F1s will be screened for the heterozygous mutation by fin clipping. Heterozygous F1s will be further outcrossed to wild type fish to segregate away any nonspecific mutations and in-crossed to other transgenic lines (mutant lines to generate double mutants, GFP-transgenic reporter lines). We will raise 100-150 F2s from these crosses and screen them for the desired heterozygous mutations. The mutant line will be then maintained as for other wild type and transgenic lines, which corresponds to about 4 tanks of around 15 fish each.

We currently maintain 28 mutant lines and 23 transgenic lines. We estimate that in the next 5 years we will generate around 8 new mutants and 20 new transgenics.

Our mouse lines are routinely maintained by keeping 2-3 breeding pairs, with around 3-4 litters/year - total 75-100 animals per strain/year. For mouse cancer models involving multiple alleles, such as the SMAD4 null KPC mouse model of pancreatic cancer, where only approximately 1 in 16 to 1 in 8 mice (depending on the exact genotype of the parents) with the desired genotype will get tumours from an in-cross, then 10 breeding pairs will be kept with 6-8 litters/year – 700 mice per strain per year.

For the mouse work, the estimate is based on several factors. Firstly, we are using our previous experience of the last 5 years and the experience of our collaborators who use similar approaches with the same mouse lines. This is a good guide as much of the work we are planning is a direct continuation of work currently on-going in the lab. Secondly, we have taken into account the number of researchers within the group who perform mouse experiments (currently 2, and likely to be no more than 3 in the next five years). Numbers of mice used for breeding are based on best practice and we currently maintain 2 strains and plan to generate one more.

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

For the zebrafish work we regularly review our mutant and other stocks and cull any that are no longer required. For all zebrafish lines, we freeze sperm to archive the line. Thus, we will only maintain lines in the aquarium that we are actually using in on-going experiments. Through exchanges with other zebrafish labs, in the UK and elsewhere, we will be able to minimise the number of mutant and genetically modified strains that we keep in our own aquarium. Moreover, the stocks of adult mutant fish that we will keep will almost all be heterozygotes carrying recessive mutations and thus phenotypically normal. When we are generating new lines or bringing in new transgenics or mutants from other aquaria, we carefully plan the experiments we will do to minimise the number of lines generated. For example, where we might want to generate a line with a fluorescent signalling reporter that we might want combine (by breeding) with a different colour fluorescent signalling reporter, we plan in advance what fluorescent readouts we should use for each reporter so we can make the desired combinations.

In our zebrafish work we perform the majority of our experiments on zebrafish embryos before they are independently feeding, and from one cross we get about 100 embryos on average. Many of the experiments will involve live imaging developing embryos which precludes any blinding. However, analysis pipelines will be automated where appropriate to reduce bias as much as possible. While live imaging and analysis are relatively time consuming, one imaging session provides data on >100 cells per image (or up to 1000 cells from in toto Lightsheet microscopy). Therefore, to ensure robustness in our studies at least 3–5 embryos will be imaged from different clutches of embryos per experiment. All experiments are performed in triplicate. For ‘omics experiments we will use 50–100 embryos per time point to acquire sufficient cells to analyse and to average out small differences between embryos.

For the mouse work experiments are designed to not falsely detect effects and not miss effects. Based on our previous experience of how variable our measurements are and how big the effect we are looking at is, then most experiments involve around 5-10 mice per group and 3-7 groups. If the necessary in vivo data does not exist, then experimental design will be informed by a combination of prior in vitro data generated in the laboratory, existing publications, and previous experience. We also continually re-evaluate the numbers of mice required for each experiment using power calculations. For this we access help from an in-house statistician at the e stablishment when necessary. This will allow us to determine the number of animals required per experiment.

A measure that we have put in place to reduce variability for experiments designed to test therapeutics on pancreatic cancer includes monitoring initial tumour development by ultrasound prior to beginning treatment so that we are sure that all mice in the groups have tumours of a similar size prior to treatment. We then monitor tumour development

throughout the experiment by ultrasound so that we have early warning of tumours that might begin to impede critical functions in the mice.

We aim to maximise the data from each experiment by fixing and embedding tumour tissue as well as organs that might be affected by the cancer so that we can perform many additional experiments downstream in vitro.

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

For the zebrafish work, we endeavour to keep the breeding as efficient as possible and keep 4 tanks of 15 fish each of established lines which gives us enough fish to get the embryos we need for our experiments and to keep the line secure. If multiple people in the laboratory are working with the same line, we coordinate experiments so that embryos do not go to waste. For the mouse work we will try to keep as few mice as possible by careful monitoring our mouse colony and good practice. To minimise breeding, lines under sporadic use are maintained at lower levels, and frozen whenever practicable. Lines will be maintained in collaboration with other licensees wherever possible to minimise redundant breeding.

From the genetically modified pancreatic cancer model (SMAD4-null KPC model), we are also generating cancer cells and cancer-associated fibroblasts which we are using for in vitro models, which will allow us to test hypotheses in vitro before exploring them in the in vivo setting. We are also moving to investigating the role of Activin and TGF-β signalling in pancreatic cancer metastasis. For these experiments we will use some of the same tumour cells that we are harvesting for the in vitro models, which will reduce the numbers of mice required. In addition, where practical, we share tissues from mice among appropriate lab members.

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 the zebrafish model for the vertebrate developmental work and for modelling the Marfan syndromes and Myhre syndrome, as these embryos develop ex utero and can be manipulated genetically and are transparent, and thus ideal for imaging.

All embryological work is preceded by in vitro or tissue culture studies to test the approach or activity of the biological entity to be introduced into the embryo. Failure at this preliminary stage is taken as final and no in vivo work will take place until this step is successful. In addition, for the zebrafish we will minimise suffering by taking pains to maintain the general health of the fish population, by attention to water quality, feeding regimes, and fish population density in each tank. We will check all breeding stock daily and cull any that show signs of significant illness or deformity. Where potentially distressing procedures are required, e.g. fin clipping, we will perform them under general

anaesthesia with analgesia both pre and post fin clip. Any fish or fish larvae showing signs of distress on recovery from a surgical or other procedure will be killed promptly by an approved method.

We will use mice in this project to investigate tumourigenesis in a mammalian system and to test new therapies. For the generation of breast tumours, we will most often employ sub-cutaneous injections or injections into the mammary fat pad. Using syngeneic transplant, we have better control of tumour growth dynamics, than using a spontaneous genetic tumour model, where tumours arise at multiple sites sporadically. This reduces mouse numbers as 1 tumour from a genetic model can be used to generate tumours in around 15 mice. We use ultrasound-guided injection into the pancreas to study pancreatic cancer. Similarly, this has advantages over a spontaneous genetic tumour model, as we have more control over the time of tumour development. For the study of lung metastases, intravenous injections will be the route of choice. On rare occasion, we will study metastasis to other organs and therefore use other injection routes, such as intra-cardiac to give bone metastases or injection into the spleen or liver to give rise to liver metastases which are relevant for the pancreatic cancer model. For skin tumours, we will use a topical carcinogen application. This is a well characterised method for efficiently generating reproducible skin tumours that does not rely on a GEMMs model. We are also using genetic models for breast cancer to mostly to generate tumours for transplantation assays. For the pancreatic cancer model, we are using spontaneous genetic tumour models. The parental lines are maintained at a minimal number and only bred in larger numbers for particular experiments.

For the mouse work we will check the animals every day and humanely kill animals exhibiting obvious signs of illness. We will also reduce the overall tumour burden on mice and thus minimise possible adverse effects, by for example using the non-invasive imaging for metastasis assays.

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

We use zebrafish embryos for our developmental work, and most of this is on fry less than 5 days old, which are deemed non sentient and before the onset of independent feeding. For the tumour work, we cannot use a less sentient model as it is important for our research to have the fully functioning mammalian immune system and organs. We also cannot use very young mice as their immune system is not sufficiently mature for our experiments. We also use terminal anaesthesia to obtain very detailed information about tumours.

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

We try to minimise any possible adverse effects. In particular, the use of fluorescently labelled cells coupled with microscopic analysis of tissues enables us to detect small metastases. This reduces the overall tumour burden needed to be able to detect metastasis from primary sites. Similar benefits are expected from the use of non-invasive fluorescent or bio-luminescent imaging. We use ultrasound to monitor the growth of pancreatic cancers that are difficult to monitor by palpation. If the purpose of the experiment is simply to observe cellular behaviours in the primary tumour, then we would not grow the tumours to the larger size that some of the metastasis experiments or experiments investigating the effects of therapies require. Where we do require tumours to grow to larger sizes we will kill the animals when humane endpoints are reached. In addition, we will carefully measure tumour volume postmortem to determine whether there

is a tumour size that consistently correlates with animals becoming unwell. If this is the case, then this volume measurement can be used in the future as a guide to determine when the humane end point has been reached. Regular monitoring by animal care staff is in place and we strive to keep updated with the latest environment improvements, such as enhanced environmental stimulation.

For the zebrafish work, the most invasive procedure we use in adults is fin clipping for genotyping. We will also use skin swabbing as an alternative to fin clipping which is less invasive. It has a slightly lower efficiency rate so we will use it for simple genotypes. For all our transgenic lines, we genotype by visualising fluorescence in the offspring, which eliminates any need for fin clipping or skin swabbing.

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

We are aware of NC3Rs and have consulted the following publication https://www.nature.com/articles/6605642 , which provides updated guidelines on the welfare and use of animals in cancer research. We will additionally consult the OBSERVE guidelines

(https://www.nature.com/articles/s41596-024-00998-w). We also discuss with colleagues in other research groups new improvements that lead to refinement.

We additionally consult the PREPARE guidelines when designing experiments https://norecopa.no/prepare/ and https://researchanimaltraining.com/article-categories/procedures-with-care/

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

We will stay up to date via regular communication with animal care staff, other scientists in the field and regular visits to the following website https://www.nc3rs.org.uk/3rs-resources.

59. Interactions of extracellular vesicles and vascular modulators on cancer development 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
  - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants

Key words

cancer, extracellular vesicles, vasculature, blood vessels, drugs

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 goal of this project is to improve our understanding of cancer so that better treatments can be later developed by:

Investigating how extracellular vesicles influence how cancer progresses and spreads.

Exploring how extracelullar vesicles and drugs interact with blood vessels in ways that alter how cancer progresses and spreads.

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 a leading cause of mortality in the UK. Nearly half of people born in the UK will develop cancer in their lifetimes (NHS). There is a need for better drugs that to combat cancer. The key to developing safe and effective drugs is to better understand what drives

cancer progression and its spread to distant organs (metastasis). Some key components that are understood to be involved in cancer are extracellular vesicles (small particles produced by both tumour cells and healthy cells that circulate through blood) and blood vessels (that serve to transport extracellular vesicles, drugs and tumour cells). The role of these components on how cancers progress and spread however is not fully understood and so this work is important because new insight into how these components influence cancer may allow us to devise safer and more effective therapies.

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

We expect that this project will generate important new scientific findings on how extracellular vesicles contribute to cancer progression and spread. This fundamental new knowledge will have impact through multiple avenues.

First, our scientific findings will be distributed through journal publications, conference presentations, patents and through in person meetings with other experts and clinicians. This will enable other researchers to advance other avenues of research that may have far-reaching consequences for further understanding this devastating disease.

Second, our work will be showcased to the public through direct interactions with the public through articles and outreach events which will educate the public on cancer, but also may help inspire the next generation of scientists and public to contribute to further research.

Third, our new scientific findings may give us insights into how new classes of drug candidates may be developed to combat cancer progression and spread. These drug candidates and their methods of production will form new intellectual property that will be secured through UK and international patent filings which will help motivate drug development in this area, for instance by serving as the foundation for new start-up companies that will eventually produce powerful new anti-cancer drugs for the greater good.

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

Impact on Academia and Scientific Knowledge:

This research will generate crucial data on how cancer responds to extracellular vesicles and modifications to blood vessels. This will enable further research in this area and into related areas of cancer. Such insights can quickly inform academic research so we expect these benefits to be realised in the short term

Impact on Patients & the NHS

This project has the potential to lead to significant benefit for the approximately 3 million patients with cancer in the UK. An understanding of the influence of extracellular vesicles and the vasculature on cancer progression could lead to novel classes of treatments for this disease. The drugs or interventions that may be developed as a result of this project may be safer, have fewer detrimental side effects, and be more effective than current therapeutic options. This may translate into longer life expectancies and higher quality of life for patients with cancer. Furthermore, the development of such interventions in the UK would tremendously elevate the status and effectiveness of NHS cancer care in the longer term. We expect that these impacts will be felt in the long term beyond the duration of this work because of how long it takes to develop, test, obtain approval and market new drug candidates.

Impact on the Economy

The results of this project have the potential to greatly improve the economic competitiveness of the United Kingdom by enabling the development of novel cancer therapeutics along with the relevant patents that would be obtained during the course of the project and beyond. The pharmaceutical industry in the UK employs over 70,000 people and contributes over £8 billion pounds to the economy. Furthermore, improvements in the overall life expectancy, quality of life, health and creative output of people in the UK would be improve with the advent of such therapies, which would translate into compounded economic benefits. We expect that these results would be realised in the medium to long term since spin out companies and further development efforts may lead to the employment of people potentially from the final few years of this license to decades beyond the end of the project.

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

We will publish our findings in fully open access journals in line with UK Research and Innovation's policy on Open Access. This will ensure equitable and broad dissemination of our findings to other experts, clinicians and the public.

Scientific knowledge will also be distributed to the public through outreach events as well as articles (websites, blogs, magazines, etc.), podcasts, and other media. We will also work with institutional communications team to create press releases and popularise our work.

New classes of investigational drug candidates will be secured as intellectual property through UK and international patents. We will then translate our intellectual property to the market via the spin-out of start-up companies.

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.

Mice are the least sentient mammalian species that is physiologically representative of many human tissue structures (e.g. vasculature and extracellular vesicles) that is widely used within the research community. The implantation of human tumour cells in mice generates tumours that have similar morphology, histopathology, and molecular characteristics to human cancers therefore adult mice models are representative models for understanding the progression of human cancers and the activity of potential therapies.

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

A typical experiment involves up to four phases: pre-treatment, disease model establishment, longitudinal monitoring, intervention, and termination.

Pre-treatment: In this phase, extracellular vesicles will be introduced into animals via injection into tail veins. This process may be repeated multiple times and some animals

may be put asleep prior to injections. Not all animals may undergo a pre-treatment phase, and may instead be given injections of saline instead.

Disease model establishment: In the disease model establishment phase, human cells will be introduced into animals via injection into tail veins, under the skin, or into mammary fat pad to create tumour lesions. The welfare of animals and the progression of the disease will be monitored hereafter at regular intervals.

Longitundinal monitoring: Starting from the disease model establishment phase and running until the end of the experiment, animals will be monitored at regular intervals. Monitoring may involve various imaging techniques such as bioluminescence monitoring to observe the distribution or growth of injected materials, or other imaging modalities such as computer tomography, magnetic resonance imaging, positron emission tomography and ultrasound. This phase will overlap with any intervention phases, but not all animals may undergo longitudinal monitoring.

Intervention: In this phase, biomolecules such as drug candidates and/or extracellular vesicles (modified or unaltered) will be introduced into animals via injection into tail veins. This process may be repeated multiple times at intervals 20 hours or longer. Not all animals may undergo an intervention phase, and may instead be given sham injections instead that contain no biologically active ingredients but will contain solvent such as saline.

Termination: Animals will be humanely killed at pre-determined timed points or earlier when it is deemed ethical to do so, so that organs and lesions can be harvested for later analysis.

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

We expect animals to experience acute pain from tail vein injections.

Depending on the cellular model and its load, animals injected with human cancer cells may experience pain and discomfort for up to approximately 6 weeks. Animals will be humanely killed if relevant signs of suffering or distress are noticed.

Animals injected with sublethal doses of biomolecules such as drug candidates or extracellular vesicles may experience some mild or moderate pain, discomfort, reduced locomotion and fatigue over short periods. Animals may experience some weight loss potential due to reduced appetite.

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: throughout all of the protocols, we estimate that approximately 85% of the mice will undergo Moderate procedures while the remaining 15% of the mice will undergo Mild procedures.

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 progression of cancer and the growth of tumours is an extremely complex phenomenon that is highly dependent on the microenvironmental conditions and supporting tissues. We are particularly interested in a) how extracellular vesicles modify and alter the tissue microenvironment in ways that may support or hinder cancer progression, and b) how the biophysical and biochemical cues, such as fluid shear stress and endothelium integrity, present in the unique microenvironment of blood vessels alter cancer progression and how these cues may be influenced by extracellular vesicles and drugs. We need animal to accurately replicate the complexity of the tissue microenvironment in which cancers develop.

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

In vitro laboratory models, including 2D cultures, 3D cultures and microfluidic devices
Computer simulations

Why were they not suitable?

In vitro laboratory models and computer simulations are unable to fully recapitulate the complex tissue microenvironment that inhibits or promotes tumour progression. This prevents us from fully understanding how a particular agent may interact within humans. For instance, we have no vitro laboratory models that recapitulate pre-capillary sphincters that rapidly respond to biochemical cues that control the diameters of capillaries vital for the entrapment of circulating cancer cells. Nonetheless, we have and will conduct experiments using tumour cells in microfluidic and other in vitro models prior to commencing in vivo work. These in vitro experiments will be particularly useful in instances where mechanistic insights are needed therefore replacing the need for some animal use. The findings of these studies will also enable us reduce the number of conditions and therefore animals needed.

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 estimates of the maximum number of animals that will be used in each protocol. This is a balance of the fewest number of animals required to explore experimental conditions while providing enough animals to draw statistically sound experimental conclusions. These are sourced based on the advice of mentors with extensive experience in the experimental design of similar animal experiments, as well as literature reports on similar animal models.

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

We defined our in vivo experimental design objectives with assistance of mentors to minimise the number of experimental conditions to be run.

Devised secondary and future objectives to maximise useful experimental information we can capture from these experiments.

Refined the experimental design through through the use of NC3R's experimental design assistant and ARRIVE guidelines.

Investigated literature reports on the design of similar protocols to identify best practices.

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 in vitro experiments, most notably microfluidic experiments, to refine animal experiments in ways that help us reduce the number of animal experiments needed. For instance, when using drug candidates or extracellular vesicles where we have limited literature reports on safe and effective doses, we will first run pilot experiments with small numbers of animals to help us identify doses that minimise adverse effects while ensuring sufficient biological activity to succeed in our experimental objectives.

After animals are humanely killed, we will maximise our harvest of tissues, organs and tumour lesions to obtain as much data as possible. Tissue samples we do not need immediately will be frozen and may be made available to other researchers if requested.

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 explore different aspects of cancer progression and spread we will use three different models. To model the progression of breast cancer, tumour cells will be injected into one or both mammary fat pads. This enables breast cancer tumours to form at the same sites as in humans (orthotopic). To model the progression of other (non-breast) solid tumours, cells will be injected under the skin

(subcutaneously) into one or both flanks of an animal. This enables tumour lesions to form close to the skin of the animals for easier monitoring. To model metastatic (spreading) tumours (both breast and non-breast cancers), cells will be injected into tail veins. This enables cells to be entrapped in small vessels and capillaries which is a key step in the spread of cancers. All three methods are highly refined with well established histories of use in cancer research.

Animals will be monitored closely for signs of distress and tumour lesions will be imaged at regular intervals after disease models are established to limit suffering and distress. This will include endpoints such that animals will be humanely killed when tumour volumes exceed set sizes or when signs of reduced body weight are noted.

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

Human cancer is a complex disease that requires a mammalian host and mice are the least sentient model that is widely used in this area. Adult mice are required since the tissue microenvironment of an immature mouse is poorly representative of those in which adult human tumours are exposed. Other less sentinent models were considered but ruled out. Drosophila are too small to facilitate human tumour lesions and are physiologically too different to draw comparisons to human disease. Zebrafish (adult or embryo) are non-mammalian and require temperatures significantly below 37C, at which human and human tumour cells are adapted. Thus, results in these models would be poorly representative of human disease in these cases.

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

We will monitor and review the welfare and distress in our animals throughout all animal experiments alongside our animal facility colleagues. Animals will be monitored closely for signs of distress and tumour lesions will be imaged at regular intervals after disease models are established to limit suffering and distress. This will include endpoints such that animals will be humanely killed when tumour volumes exceed set sizes or when signs of reduced body weight, abdominal palpation or loss of condition are noted.

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

We follow the LASA, ARRIVE, and PREPARE guidelines and will consult with NC3R resources such as the experimental design assistant to continually refine our practices. We also use the latest published literature on best practices such as the OBSERVE: guidelines for the refinement of rodent cancer models (De Vleeschauwer et al) and the Guidelines for the welfare and use of animals in cancer research (Workman et al.)

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

A 3Rs officer will be assigned in the laboratory whose role is to seek improvements to 3Rs. Animal work and the 3Rs will be regularly discussed in laboratory meetings and we will work with our animal facility to implement the best practices, improving our 3Rs. We also review the literature to identify the best 3Rs practices and through NC3Rs monthly and Tech3R newsletters.

60. Embryonated Eggs for Diagnosis and Research

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

Virus, Vaccine

Animal types	Life stages
Embryonated Chicken Eggs	Embryo and egg

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 will provide support to work to diagnose, monitor and study viruses causing human infections.

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?

Influenza epidemics are associated with high levels of illness and death. Diagnosis of influenza infection provides a public health benefit by providing information on the number of virus infections in the population. This data can also be used to inform how well matched the vaccine is to the virus infecting the population and also how well the vaccine is performing in protecting the population.

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

Outputs will include virus strains and stocks that can be used to support generation of data on how well matched the vaccine is to the virus infecting the population and also how well the vaccine is performing in protecting the population.

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

In the short term the outputs support the work of the group which provides a long term public health benefit in terms of diagnosing and monitoring viruses causing human infections.

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

Outputs of the work will be used to support scientific studies which will be published or shared as reports with collaborators. Data from these studies is provided to other researchers and can be used to inform how well matched the vaccine is to the virus infecting the population and also how well the vaccine is performing in protecting the population.

Species and numbers of animals expected to be used

- Embryonated Chicken Eggs: 1750

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.

Embryonated eggs have been used for decades as a highly efficient means for growth of human viruses for research and for vaccine development and production.

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

Embryonated eggs at mid-incubation are inoculated with a carefully prepared virus dose and incubated for a short time period, typically 2-3 days. The embryos are terminated at the end of the procedure and before hatching. Embryonated eggs are typically used within the first two-thirds of the gestation period.

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

Expected adverse effects are from microbial contamination during the procedures. This is minimised by ensuring sterility of equipment, careful monitoring of the procedures, quality control and staff training and competency.

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 is 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?

Some viruses will only grow in embryonated eggs. Some diagnostic tests require high concentrations or volumes of virus which can only be achieved by growth in embryonated eggs. Many influenza vaccine viruses are produced in eggs. Diagnostic testing associated with the influenza vaccine must use egg grown viruses to preserve the characteristics of the vaccine virus.

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

In vitro methods such as tissue culture cells are routinely used for growing viruses and this method is frequently selected when it is the best approach. However in cases where the required viral antigen must be a close match to the egg grown vaccine strain, the use of embryonated eggs to generate virus stocks is necessary.

Why were they not suitable?

Growth of viruses using different methods such as in eggs or tissue culture cells exerts selective pressure on the virus which may mutate in response. This is often due to the presence of different structures on the surface of the cells which the virus uses to gain entry into the cells. These can differ between cells found in embryonated eggs and in different kinds of in vitro tissue culture models. Mutations in the virus in response to growing in these different environments can have a knock-on effect on how closely the virus resembles the vaccine and this must be carefully considered when selecting growth methods so that the virus stock produced is suitable. While vaccines are still produced in eggs, the requirement for matched virus stocks for vaccine studies will be necessary.

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 estimate takes account of a regular supply of eggs which are incubated under the correct conditions to ensure consistent availability. Availability of suitable eggs at the correct gestation stage can only be guaranteed with a regular supply. This is kept to the

minimum level required. Many years of experience has shown the yield of egg fluid that can be obtained from a batch and the expected wastage. The minimum number of eggs is used to ensure the experiment will provide a sufficient volume of high titre virus. Regular egg availability is required as viruses often have to be passaged in fresh eggs immediately after harvest.

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

Wherever possible, tissue culture cells are used instead of embryonated eggs. When eggs are used the quantity is carefully selected according to standard operating protocols to ensure that the aim of the experiment is achieved without unnecessary wastage. The number of eggs to be used is determined by the use of standard protocols to ensure the quality of the scientific outputs of the work. During the term of a previous licence the regular consignment was reduced by 30% to reflect refinement in the process and an overall reduction in the number of eggs used.

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 are used with a new virus strain to determine the optimum concentration to use ahead of a larger scale study. Conditions used by laboratories using the same virus strains will be taken into consideration when planning the experiment. Records of previous experiments are also available to guide work with new virus strains.

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.

Embryonated hens' eggs have been used for many years for growth of viruses for use in vaccines and vaccine development. Procedures are well established and have been refined over time following standard operating protocols and routine procedures. Eggs are housed in dedicated incubators which are regularly monitored for humidity, temperature and rotation. Incubator temperature is monitored continuously with a 24/7 temperature monitoring system. Microbiological Safety Cabinets (MSC) are used for inoculation of eggs to protect the worker and mitigate against contamination.

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

Use of eggs is restricted to purposes where they provide a significant benefit which cannot be achieved by the use of tissue culture cells.

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

Eggs are carefully checked immediately on receipt after hatching and any that are unsuitable are discarded at this stage prior to incubation to develop the embryo. Following the incubation period eggs are again carefully checked and any that are unsuitable for the experimental procedure are terminated. This ensures that only suitable eggs are incubated and used for the procedures. Eggs are handled with care by trained and experienced staff, housed in dedicated incubators and carefully transported once received within the facility to minimize any harm.

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

Training and sharing of protocols with other laboratories and networks, and reference to resources such as the PREPARE guidelines, ensures that methods are updated and refined to achieve the best result.

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

By seeking training and guidance, by attending relevant training courses and by regularly checking information on NC3Rs website.

61. Model development for oncology drug discovery.

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

tumour models, medicine discovery, cancer therapy, in vivo pharmacology, tumour microenvironment

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 purpose of this Project Licence is to develop and establish new models to support the discovery and development of new medicines to treat 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?

In 2022, as published by the World Health Organisation, there were an estimated 20 million new cancer cases and 9.7 million deaths. In UK alone according to Cancer Research UK data, over the last decade (between 2006-2008 and 2016-2018), the cancer incidence rates increased by 4% and they are projected to rise further by 2% in the UK in the next 10-15 years to come; meaning that many more cases of cancer will develop in patients which will not be merely due to age.

Despite the significant advancements in treatment of cancer over the last two decades, there are still many types for which tumour-targeted therapies are not available. In other cases, such therapies are available, but treatment is not effective for all patients and many

patients who do respond to these therapies will eventually develop resistance and their tumour grows back. Of those patients diagnosed in the UK, only 50% will survive for 10 years or more following their diagnosis.

In this Project Licence we aim to develop and establish new complex in vivo models for cancer therapeutics that are expected to better mimic the target organ biology of the patient. These new models will complement the existing models already used to study the mechanisms involved in cancer development to enable the development of new medicines for cancer patients The intention of this PPL is to build models, such as colorectal orthotopic, but may in the future diverge from orthotopic tumour models to include models for colitis or infection protocols. Our intention is to design separate protocols of each new model that would go through internal approval process before being added to this project.

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

This work will help establish complex models, like orthotopic tumour models, colitis models or infection models to name a few, that will be used to advance our experimental cancer medicines through multiple phases of research and development by evaluating their activity in animal models of disease. These models will mimic patient biology more accurately and thus will help us to develop an insight into whether the new medicines work in more relevant clinical settings and possibly could also enable us to identify potential targets for new medicines and thereby expand treatment options for cancer patients.

Equally, use of complex models developed under this project work may also guide us to determine which of the experimental medicines should not be progressed further. In addition to the outputs mentioned above, we expect to publish our results and to present our findings externally to scientific peers.

We expect that such more patient relevant models which reflect human disease more accurately may reduce the use of traditional, less reflective models, thus reducing the number of animals used for experimental work as they will be more accurate to predict the drug outcomes to enable decisions whether the new medicines can be progressed to the clinical trials in relevant disease indications.

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

This programme of work is expected to enable us to develop patient-relevant disease models. Models established using this licence will be used to test and progress new cancer medicines which, if successful, will have the potential to benefit millions of cancer patients across the globe in the longer term. However, as the discovery of the new medicine is a slow process, in the short term these models will help progress the right format of the ‘candidate medicines’ through the discovery pipeline. These candidate molecules, if approved for use in the clinics, will then be available to treat patients with cancer who otherwise are likely to die from their disease.

The development of new or improved cancer models will help us to target the right patient populations with our medicines which will increase the likelihood that those patients will benefit from the treatment.

The work carried out under this licence is also expected to contribute knowledge to the broader scientific community through the publication of our findings and presentations at internal and external scientific meetings on regular basis.

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

Our team has an excellent track record of publishing the advances in discovery of new medicines as well as animal modelling. We aim to publish both successful and unsuccessful experimental results in relevant scientific journals, and to share our data and learnings with numerous collaborators to avoid duplication of research work and efforts. We present our research at national and international conferences for the benefit of the broader scientific community. This public dissemination of our results has the potential to lead to new collaboration and opportunities to develop new innovative experimental cancer models and medicines as well as to further characterise or find new associated indicators of disease (biomarkers).

Species and numbers of animals expected to be used

- Mice: 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.

Mice are one of the most commonly used animals for biological research, especially cancer research. This is because many more tumour models are available for mice, and a wealth of published data exists to help guide the experimental plans and interpret results. This is also true for tumour immunology studies. The immune system of mice resembles that of humans in many respects which means that tumours can be grown in these animals to create patient-specific models to investigate new experimental cancer medicines along with their effect on the immune response to tumours.

This research project will use mice to support our efforts to discover and develop novel therapies for cancer to benefit these patients. Specifically, we will use mice to enable us to mimic the complex aspects of the disease as well as the properties of the medicines that are currently not possible in a laboratory setting or by using simplistic research models. It is important to undertake this program of work to support the development of new formats and technologies that are being developed for cancer therapy and test them in a complex live animal model that imitates the nature of the disease. This program of work will establish models which will be used by the team to progress new cancer medicines through the discovery and development pipeline.

We will also use immunocompromised mice; these are mice that have defect in their ability to produce an immune response which allows human samples to grow in them without rejection. We use models termed as 'humanised'; by injecting both human immune cells as well as human tumours to model specific aspects of the disease that we cannot do in 'mouse only' systems.

Typically, we plan to use adult mice (6-14 weeks old) at the start of our studies as the immune system is considered immature in younger mice and would not represent the biology of the cancer patients we aim to treat.

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

Animals are kept in high-quality facilities, free from pathogens (disease-causing organisms such as bacteria, viruses, and parasites) and with access to food, water, and environmental enrichment. In all the facilities used, the animal care staff are highly trained

in rodent welfare and will ensure the animal suffering is minimised. Animals are housed in groups except in exceptional circumstances, for example when aggressive behaviour puts the welfare of the animal at risk or when cage-mates have been removed for experimental reasons.

Most of the animals will be used for studies to create tumour models that better recapitulate (mirror and predict) the clinical circumstances and/or patient disease patterns. The vast majority of tumours will result from either surgical implantation or specialised injection of tumour cells or tumour or selforganising 3D tissue cultures (so called organoid) fragments into a specific organ (e.g. cecum or colon).

In most studies, we will use advanced imaging techniques (such as bioluminescence imaging, ultrasound etc.) that will allow us to monitor the tumour growth. To undertake such imaging sessions animals will be anaesthetised so that consistent and reproducible measurements could be taken in a motionless, sleeping animal.

Blood samples may also be collected during some studies to measure levels of cytokines or medicine, or human immune cell components in humanised models. Small volume, max 10% of total blood volume in a living animal at any one given time-point or 15% of total blood volume in a 28 day period, blood samples are taken from a vein while the study is ongoing, whereas larger blood samples are taken at the end of study if a greater volume (usually total blood is collected) is needed. Such a procedure will always be carried out at the end of the study under a non-recovery anaesthesia.

To establish and characterise the new tumour models we may also inject medicines, such as those used as standard of care in patients like chemotherapy or our own experimental cancer medicinecandidates. Medicines are commonly injected into the peritoneal cavity (i.p.; into the abdomen), intravenously (i.v.; into the tail vein). Occasionally medicines may be administered orally (oral gavage; o.g. or p.o.) or under the skin (s.c.; subcutaneously).

At times medicines may only need to be administered once, but more often they may be administered according to a schedule that requires multiple administrations. The administration schedule is usually determined by the properties of the medicines like their effectiveness, stability inside the living body etc. For example, medicines injected into the peritoneal cavity are typically given two to three times per week, whereas those given orally would typically be administered once or twice a day, often for the duration of the study. In most cases our studies last approximately one to two months but on occasion when tumours grow slowly, they could last for 6 months.

Following is an example of typical steps in a study with an immunocompromised host animal to develop the orthotopic colorectal tumour model (Protocol 1):

implantation of a microchip under the skin (s.c.) for identification

injection of human immune cells into the tail vein (i.v.)

surgical implantation of tumour cells into a target organ (e.g. cecum or colon)

tumour measurement/ monitoring

administration of experimental or standard of care (SoC) medicine by one or more of the following routes: i.p. into the peritoneal cavity (typically this will be done twice a week for three weeks); intravenous i.v. into the tail vein (typically this will be done once or twice a

week for 3-4 weeks); orally (by gavage; (typically this is done once or twice a day for the duration of the study)

collection of a blood sample(s)

At the end of procedures, all animals will be humanely killed. Tumour, blood and other organs may be collected for any analysis.

The steps in a typical study involving the non- immunocompromised host animal will be the same as above except for step 2 which will not be needed.

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

In these studies, the likeliest sources of adverse effects are from surgical procedures, size and condition of the tumour, or in humanised models from graft versus host disease (GvHD). “Graft” here refers to transplanted human immune cells “host” refers to the animal.

Animals will undergo surgical procedures for tumour implants and are expected to experience pain due to the surgery and will be classified as moderate severity. Anaesthetic and pain relief (analgesia) will be provided when surgery is performed as per veterinary consultation.

GvHD is a systemic disorder that occurs when the graft's immune cells recognize the host as foreign and attack the recipient's body cells. Animals will be classified according to a scoring system that is in place and is based on the degree and duration of clinical observations such as body weight, activity level, posture, and body condition.

We will humanely kill any animals that have developed large tumours or have developed tumours that are impeding any movement or leading to significant abdominal swelling due to fluid accumulation to minimise unnecessary suffering.

At times, use of genetically modified mice is necessary for our work. Some of which will be immunocompromised mice, such mice may experience hock swelling. Mice will be assessed, using a score sheet, based on the size, capacity to grip, presence/absence of superficial lesion and gait. Affected mice may be given altered enrichment and/ or pain relief and/or anti-inflammatories in consultation with the veterinary advice. Mice that are unable to grip, reluctant to move or are affected bilaterally, with associated gait disturbance, will be humanely killed. Any treated mice that do not respond within 10 days of treatment onset will also be humanely killed.

Clinical signs will be seen in majority of the animals undergoing procedures in this Project License. Typically, clinical signs will be moderate in severity due to involvement of the surgical intervention.

Animals are expected to recover well from surgery before they are enrolled for any drug treatment. From experience, clinical signs will include gradual weight loss of up to 20% from maximum weight, partial to marked piloerection, transient to intermittent hunching in the posture, subdued (toned down) but responsive behaviour, abnormal walking, short term ataxia (uncoordinated movements), intermittent diarrhoea. Some animals may also develop paleness in extremities.

Treatment of animals with cancer therapies may also lead to unwanted effects like those experienced by human patients. While humans may experience fatigue or fever soon after

receiving the therapy, we observe similar responses in mice such as reduced mobility, hunched posture, and piloerection (bristling of fur). Most of these effects will be mild and of short duration; however, some animals may experience moderate effects like, gradual weight loss of up to 20% from maximum weight, partial to marked piloerection, transient to intermittent hunching in the posture.

For such animals we'll have monitoring and control measures in place such as dosing animals early during working hours and observing animals for any adverse reactions and if animals are rapidly losing weight, then supportive measures such as wet mashed food or diet gels will typically be provided.

At the end of procedures, all animals will be humanely killed and humane endpoints will be applied under veterinary guidance as 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)?

Moderate; 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?

This research project will use animals to support our efforts to discover and develop novel therapies for cancer to benefit these patients. Specifically, we will use animals to enable us to mimic the complex aspects of the disease as well as the drug properties that are currently not possible in a laboratory setting or by using simplistic research models. It is important to undertake this program of work to support the development of new drug formats and technologies for cancer therapy and test them in a complex live animal model that imitates the nature of the disease. This program of work will establish models which will be used by the team to progress new cancer medicines through the drug discovery and development pipeline.

We do conduct numerous in vitro experiments in the laboratory using cells, 3D tissue cultures (organoids mixed culture of cells of different origins and types; use molecular biology, and computer simulation modelling techniques to answer some of the questions, but it is still necessary to use some animals for research so that we can more accurately model the biology of the disease as well as the interactions of cancer cells with other cells and organs within a living body.

Isolated mixed culture of cells of different origin or 3D cell culture do not reproduce the complex nature of in vivo (in a live animal) biology. The use of animals will allow us to understand cancer in the organ of origin or as it spreads throughout the body. The most crucial aspect of our work is to understand the biology of the disease in an 'as it is' settings

that resembles the patient situation more accurately, and then to extend this understanding in discovering the biology of different immune cells to harness them to attack tumours.

And it is not possible to fully recreate such complex system and interactions outside of a living animal. Thus it has to be a combination of in vitro and in vivo studies that provide the insight needed to understand cancer biology and develop new therapeutic approaches to treat it.

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

Our department regularly uses computer modelling to fine tune the discovery of experimental medicines. A range of in vitro experiments are undertaken in laboratory dishes, containing single cell type or multiple cell types cultured together, which allows to study both direct interaction and indirect communication among different cell types. These are well-established 2-dimensional experiments that are useful to understand the specific ways that our experimental medicines affect tumour cell function.

We are now also regularly using more complex 3D experimental systems, such as patient-derived organoids and/or tumour slice culture system (TSC). In such system, the biological samples donated by patients are collected and processed (e.g. cut into thin slices in case of TSC) and then cultured in vitro. These methods preserve the spatial architecture and/ or the 3D structure of a patient tumour and are believed to be more representative of the biology of the whole tumour compared with the 2D experiments mentioned above.

Such experiments can only be undertaken for short durations of time due to practical considerations and/or viability of the patient material, however they are useful to find the lead experimental medicines that can be tested in animal models.

Why were they not suitable?

Cell-based methods are useful to gain an understanding of the way that experimental medicines impact the function of different cell types outside the body but cannot sufficiently demonstrate whether the cancer medicines remain stable after they enter the body, can reach the site of the tumour, or whether they are capable of inhibiting tumour growth in a live animal.

The 3D assay systems setup using the samples donated by patients (e.g. tumour slice or organoid cultures) are valuable additions to our experimental toolbox, however they have a short lifespan and can show modification of cellular functions but may not demonstrate that this effect can shrink a tumour.

None of the alternatives can demonstrate how the experimental therapies will behave in an organ specific pathological setting where biology could be very different due to numerous reasons like dysregulation (change in behaviour) of genes or due to impenetrable stroma (dense boundaries formed by some specific cell types and/or other components) in the tumour microenvironment, to name a few. The relatively simplistic in vitro experiments also cannot provide information like how the experimental medicines might be broken down by the body nor they can allow identification of specific signals produced in the body which can be linked to tumour growth inhibition or relapse.

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 estimate is based on the way we anticipate running this model. Typically, few pilot studies will be run to establish surgical techniques and model conditions. Once these are established then validation studies will be run before the model can be run in the efficacy studies. The estimated number includes how many animals per study, how many studies of each type and some allowance for anticipated issues or changes in demand.

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

Our organization has a "Good Statistical Practice" program instituted by a team of Biostatisticians where animal experimental designs are formally reviewed before they start. This review is conducted by a researcher proposing the experiment and a supporting statistician team to ensure the goal, experimental design, and data analysis align. This process ensures the design is robust and that the data can be statistically reliably interpreted to answer the scientific question. In this process of design, it is ensured that elements of blinding and randomisation are also included, where possible. Animals are typically randomly assigned to the cages.

Blinding: All studies are blinded at the point of measurement. But because we work with experimental medicines that can have transient side effects, we need to be able to identify agents dosed and therefore the whole pipeline will not be blinded.

Randomisation: Randomisation is typically conducted based on body weight or tumour volume of the animals. A custom-built tool is generally be used to randomise animals to groups using minimisation (Altman and Bland 2005).

We also collaborate extensively within university and outside and use practical learnings from experience of such collaborators before and during setting up of the pilot experiment for new models.

Moreover, we also use the following guidelines and online tools while designing our animal studies:

The NC3Rs Experimental Design Assistant, found at https://www.nc3rs.org.uk/experimentaldesignassistant-eda

The PREPARE Guidelines, found at https://norecopa.no/prepare

UK Co-ordinating Committee on Cancer Research (UKCCCR's) Guidelines for the welfare and use of animals in cancer research, updated in this paper: Workman et al. British Journal of Cancer; 2010: 102, 1555–1577.

OBSERVE: guidelines for the refinement of rodent cancer models (De Vleeschauwer et. al., Nature Protocols; 2024: 19, 25712596).

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

Small scale pilot studies will be carried out for each new tumour models establishment which then would be used to design next studies using the minimum number of animals needed to achieve the scientific objectives. We also implement innovative study designs to reduce animal numbers where possible. We will analyse tissues from previous experiments and from collaborators whenever possible (e.g. to confirm the expression of target gene before selecting the appropriate tumour model for our experiments). We will avoid repeating experiments for additional data collection by collecting a wide range of tissues at the time of euthanasia. And we will aim to disseminate negative results from our studies i.e. cases where compounds or models were not effective, to inform the wider scientific community. This will reduce the need to repeat experiments in the future.

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 mimic the organ specific features of a particular tumour type, we have to use complex experimental models where tumours may often develop inside the body of the animal. Such complex models may involve surgical methods to implant tumours, either in a cell suspension form via injection or that of tumour fragments.

Where possible, minimal invasive surgical methods will be used for implantation and imaging techniques to monitor tumour size. Such methods of tumour implantation are less painful for the animals and the distress caused is usually temporary that goes away in a couple of days. Methods will be chosen and put in place to allow the animals to be as mobile as possible, high standards of enrichments will be provided and the animals will be group housed wherever possible. Prior to start of any surgical procedure, we will agree with a Veterinary surgeon what pain relief medications or antibiotics are appropriate, both before and after the surgery. When recovering from surgery, we keep animals warm and monitor them closely until they display normal behaviours. They will be monitored closely on daily basis before they go on study. Where possible, appropriate imaging methods will be used to monitor tumour formation and progression.

We have also established methods like fine needle aspiration method which enables to sample tumours like similarly to biopsies taken from patients, and thereby reducing the need to run separate pharmacodynamic studies where possible.

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

The mouse is the model organism that resembles humans most closely. The human and mouse genomes are almost of the similar size, and display an equivalent number of genes, which are functionally conserved. They are the least sentient species whose physiology is very similar to humans allowing us to adequately study the complex biology of human cancer and immune system and their interactions. Because many of our experimental therapies are designed to impact the immune system, it is essential that we use animal models that will be instrumental to generate data which can be directly translated in meaningful design of clinical trials later. Currently less sentient model

animals, like C. elegans, Drosophila or Zebrafish, are not suitable for research that involves interactions among different cell types (cancer cells, immune cells) of human original especially if we also want to model the organ specific biology of the disease for such interactions.

Our studies also monitor the growth of a tumour over a period of weeks to months therefore it is essential that the animals are conscious as the use of anaesthesia (an agent that induces a state of unconsciousness) would not be possible for such an extended period. In addition, the behaviour of conscious animals also often alerts us to adverse reactions to our therapies.

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

We are committed to refining our procedures to minimise harm to the animals and have a track record of doing so. We ensure small-scale pilot studies are carried out for establishing new tumour experimental methods, or therapy testing. We carefully monitor tumour burden including the use of whole-body imaging techniques when possible. We have implemented innovative study designs to reduce animal numbers and enhanced health checks to minimize suffering. General welfare of the animals is assessed by checking body weight and watching for the development of clinical signs such as activity levels, appearance of the coat, posture, and body condition.

We are constantly refining our surgical implantation techniques including that for patient-derived xenograft (PDX) tumour fragment passage. We are currently implementing the use of a trocar as a means of minimally invasive implant (a trocar is a veterinary device that can be used for implantation of small fragments of tissue subcutaneously without the need for wound closure. We are constantly refining the use of analgesia and anaesthetic regimens with constant inputs and discussion with Veterinary surgeon and animal welfare officers. We have developed a GVHD (graft versus host disease) scoring method that we are constantly refining to ensure welfare and scientific objectives are well balanced. We also intend to develop and continually refine separate scoring systems for each new complex model that we set up as such models may lead to new adverse effects that are outside the general clinical signs usually seen. Where possible we combine multiple experimental medicines that are to be injected via the same route at the same time, to minimize the number of injections administered to the animals. When unexpected events occur, these are thoroughly investigated to find out what happened so action can be taken to prevent a reoccurrence.

Animals may also be given extra bedding or enrichment to help support wellbeing and will be provided warmth during the surgical procedure and recovery phase thereafter. Animal losing weight approaching 8% of top recorded body weight will be provided food supplements like diet gels and/ or wet mashed food.

Hypodermic needles will always be discarded after a single injection so that blunted needles (which can cause unnecessary tissue injury and pain) are not used.

For tamoxifen usage, an internal guidance document developed by the Veterinary surgeons in the establishment will be followed.

Animals imported from outside the establishment will have a standard acclimatisation period as set by the establishment. The acclimatisation process is the period during which newly arrived research animals are allowed to fully recover from stress of transport to

adjust to the new surroundings, feed, light/dark cycles, cage mates etc. Currently these are set at 7 days for national shipments or 14 days for international (overseas) shipments.

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

Our practices follow the ARRIVE guidelines developed by NC3Rs for publication of our work in peerreviewed journals (ARRIVE Guidelines version 2.0 released in July, 2020 can be found at https://arriveguidelines.org/).

Our practices incorporate many of the guiding principles of the PREPARE guidelines (Smith et al., PREPARE: guidelines for planning animal research and testing. 2017. Laboratory Animals). LASA (Laboratory Animal Science Association) also has a range of published guidance documents with principles that can be applied to our animal studies which are found at https://www.lasa.co.uk/current_publications/

Our team also closely follows the advances and best practices in 3Rs through the NC3Rs and establishment websites and via participation in conferences and events sponsored by organizations such as IAT (Institute of Animal Technology), LASA (Laboratory Animal Science Association, or NC3Rs about the best practices and refinements in the surgical as well as other procedures/ practices involving our in vivo work.

We also follow and incorporate well recognised and published guidelines to refine our workflows and best practices, like- UK Co-ordinating Committee on Cancer Research (UKCCCR's) Guidelines for the welfare and use of animals in cancer research, updated in this paper: Workman et al. British Journal of Cancer; 2010: 102, 1555–1577; as well as the latest published guidelines: OBSERVE: guidelines for the refinement of rodent cancer models (De Vleeschauwer et. al., Nature Protocols; 2024: 19, 2571– 2596) for tumour burden monitoring, expected adverse events and for selecting the appropriate humane endpoints based on overall clinical signs including tumour burden.

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

Our organisation is committed to the 3Rs and continues to follow advances in the community. We routinely hold Symposia and invite external speakers to talk on these topics and we have a dedicated 3Rs contact who regularly disseminates information related to 3Rs.

Our team is actively involved in promoting the 3Rs and participates in global 3Rs activities including an annual poster competition. This award was given to individuals working on the development of alternative models e.g. a team working on an ex vivo (taken directly from a living organism) tissue slice culture system in 2017.

Our team also is aware of advances in 3Rs through the NC3Rs and establishment websites and via participation in conferences and events sponsored by organizations such as LASA (Laboratory Animal Science Association, IAT (Institute of Animal Technology) or NC3Rs.

We as organisation are also actively seeking to adopt and implement New-Approach Methodologies (NAMs) and Non-Animal Technologies (NATs) where such methodologies are available for the scientific purposes that align to our projects.

62. Pancreaticobiliary Cancer - Investigating biology and options for therapy

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

Pancreaticobiliary cancer, Tumour microenvironment, Immunology, Therapy

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 aim of this project is to investigate the biology of pancreatic cancer development and progression in mouse models to understand the disease better and find specific targets for therapy. The models we will use will allow us to perform trials of novel therapies in these clinically relevant mouse models of pancreatic 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?

Pancreatic cancer is the 4th most common cause of cancer deaths in the western world, and is predicted to be the second most common cause in the coming decade. Current therapies are largely ineffective, meaning that only 7% of patients survive 5 years following diagnosis. This situation has remained virtually unchanged for the past 50 years making pancreatic cancer a huge unmet need.

A distinguishing feature of the disease is the dense microenvironment, almost like a healing wound, that surrounds and supports the tumour cells and can account for up to 90% of the tumour volume. This environment comprises fibroblasts (wound healing cells), pro-tumour immune cells, blood vessels, and structural proteins like collagen and fibronectin. All these components play an important role in disease progression, and can promote tumour cell proliferation, survival and spread, evasion of the immune system, and resistance to chemotherapy. Because of this, it is essential to investigate the biology of pancreatic cancer in animals, in spontaneous tumours with a physiological microenvironment and immune response.

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

We aim to increase our understanding of how pancreatic cancer develops, progresses, and spreads to other sites in the body. We also aim to find new ways to target the disease therapeutically and understand how we might best treat patients. Our project concentrates on pancreatic cancer, with some work on bile duct cancer too (the bile duct carries bile from the gall bladder to the gut where it meets the main pancreatic duct which carries digestive enzymes to the gut), but our work could apply to many cancer types. Outputs should include new genetically engineered mouse models of pancreatic cancer that represent different patient populations, findings that lead to new ways to detect or treat pancreatic cancer, and determination of the effectiveness of new therapies or therapeutic combinations that can direct new clinical trials. It's important to test treatments to show that they work before treating patients. It is likely that less than 50% of new therapies or combinations tested will be effective in the mice, however, it’s also very important that only the clinical trials that are likely to offer significant patient benefit go ahead and our work in these clinically relevant models will prevent ineffective drugs progressing to trial. Our findings will be published in peer reviewed journals.

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

Studies from this project licence should provide insights into pancreatic cancer biology, and this will benefit the rest of the pancreatic research community in the short term. Our studies will enable us to test novel targeted anti-cancer therapies in mouse models that mimic the human disease, so longer term, we hope that our work benefits patients and their families, and the medical professionals treating this disease. We will share what we learn with the rest of the scientific community by publishing our findings in scientific journals and giving presentations at scientific conferences both in the UK and internationally. We will also communicate our findings to the public through open evenings in our establishment, and meetings with supporters, patients and advocates in our establishment and around the country.

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

We will continue to collaborate with other groups involved in cancer research, particularly pancreatic cancer, and disseminate our knowledge (both positive and negative) to the community. New findings arising from these studies will be submitted for publication in peer reviewed journals and presented at national and international meetings. We will also disseminate our findings to the public through meetings with supporters, patients and advocates. We will continue to engage with the Pancreatic Cancer UK Research Involvement Network and with Cancer Research UK supporters. The licence holder and her team are active participants in charity outreach events across the country. **Species and numbers of animals expected to be used**

- Mice: 60,000

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 will be using mice that have been genetically engineered to be susceptible to developing pancreatic cancer as adults. Adult mice are excellent models in which to study pancreatic cancer for several reasons. We have a great deal of knowledge of the genetics of mice, and genetically altered mice carrying many of the gene mutations found in human pancreatic cancers already exist, meaning that we can combine these genetics quite easily. Further, the pancreatic tumours that develop in our mouse models go through the same stages of tumour progression that we see in patients, and spread to the same organs as in humans. Finally, pancreatic cancer is an incredibly complex disease that has a dense microenvironment, almost like a healing wound, which surrounds and supports the tumour cells and can account for up to 90% of the tumour volume in human pancreatic cancer. All the parts of this environment play an important role in disease progression and help tumours protect themselves from therapy. Importantly the pancreatic tumours in our mouse models also feature all these components and are just as difficult to treat with drugs.

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

Most mice in the project will not suffer any adverse effects because they will not express all the right gene mutations to develop pancreatic cancer. Of the mice carrying the right gene mutations, most will develop pancreatic cancer as adults and be humanely killed without additional procedures when they show signs of pancreatic cancer (swollen belly, loss of muscle and fat around the haunches).

Some mice will be used in preclinical trials. That means that they will be monitored for tumour development by imaging (PET-MRI, fluorescent imaging, but most commonly ultrasound) under anaesthesia (typically for less than 10 minutes). Some of these mice will have their tumours biopsied by keyhole surgery under anaesthesia with pain relief in their drinking water pre- and post-surgery. They will then be enrolled on a preclinical trial. The route and schedule of drug administration will depend on the drug, but they might be injected with anti-cancer agents from twice daily, to weekly). They will also be imaged weekly (again, usually by ultrasound). They will be humanely killed when they show signs of pancreatic cancer, which is usually ~3 weeks after being enrolled on trial, unless the therapy is effective, in which case time on treatment will rarely be longer than 2 months (<10% of mice).

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

Most mice will not experience any clinical signs, because they will not express all the right gene mutations to develop pancreatic cancer, however some mice will develop pancreatic cancer. Mice with pancreatic cancer appear to be a lot like humans with pancreatic cancer, in that they don’t exhibit any discernible signs until very late in tumour progression. This means that they may exhibit mild signs for ~5 days, and moderate signs for up to 24 hours before being humanely killed. Signs are most often a swollen belly, and loss of body condition around the haunches and this could be apparent for 3-5 days. With late stage cancer, occasionally mice may develop jaundice (a yellowish hue to the skin) or may become less mobile, hunched and/or exhibit a scruffy coat (usually not more than 24

hours). At this point they will be humanely killed. When mice undergo surgical biopsy or surgical implant of tumours there could be transient discomfort (~2 days) and this will be alleviated with pain relief.

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 the animals used will be mice. The expected severities are:

~70% sub-threshhold (those mice that do not express all the right gene mutations to develop cancer)

~10% mild (those mice that are humanely killed at early time-points before displaying any clinical signs)

~20% moderate (predominantly mice that develop end-stage pancreatic cancer)

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?

Pancreatic cancer is a very complex cancer that involves several different cell types e.g., cancer cells, wound healing cells, immune cells, blood vessels, all within a particularly dense environment. This environment, and all these different cell types, play a major role in tumour development, growth and spread, and stop drugs from working properly. Non-animal models cannot reproduce this situation and are not appropriate for studies to understand pancreatic cancer progression or for testing of new anticancer drugs.

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

We use cell line and 3D cell culture/organoid experiments, as well as archival mouse and human tissue samples where possible. We are also collaborating with a computational scientist in our establishment to develop computational models of treatment response in pancreatic cancer, using learnings from our previous animal studies.

Why were they not suitable?

Cell line and organoid systems allow us to test the effects of drugs, or of deleting/activating cancercausing genes in cells in plastic dishes but they invariably failed to deliver results in terms of predicting success in clinical trials in patients. Indeed, many clinical trials based on results from these systems have failed, with the reasons for failure subsequently demonstrated in mouse models. (It's important to note that preventing negative clinical trials from being carried out in patients is as important as driving successful trials). On the

other hand, immunotherapy, which has been a game-changer in cancer research was developed almost entirely from research in mouse models.

Cancer cells evolve and adapt to survive, and it is becoming increasingly clear that once removed from the supporting environment in which they normally grow, they 're-wire' many of their signalling pathways and no longer represent the tumour from which they came.

Archival tissue samples give us information about different mutations or altered signalling pathways, but they are not suitable for most work and any hypotheses are speculative without testing 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?

Many of our strains have predictable characteristics, in terms of cancer development and progression, and this allows us to be quite accurate in the estimation of mouse numbers we need for each experiment. However, we use very complicated genetics in our models so that we need to breed many mice to generate enough mice carrying all the genes necessary for each experiment. The numbers are based on these factors, as well as the number of genes and pathways that we plan to investigate.

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

We will minimise numbers in a few key ways, informed by use of the NC3R's Experimental Design Assistant:

Ensuring we use the fewest number of animals to show a significant response by using appropriate statistical tests. We have a dedicated bioinformatician within our lab who we continually consult to ensure best practice in experimental design.

Getting as much information from every experiment by imaging tumour progression and taking multiple tissues, e.g., pre-treatment or on-treatment biopsies also allow us to reduce the number of mice used for timepoints

Making sure we don’t breed more mice than we need

Developing ways of answering questions in non-animal models where possible

Where possible utilising less complicated genetic systems which require fewer mice to be bred

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 set up matings with the optimal genetic backgrounds to get the highest proportion of usable mice wherever possible, and where possible we minimize numbers of controls by extrapolating from different cohorts.

We will share tissues with collaborators and within the lab to get as much use as possible from every mouse.

When embarking on a new approach we will use small groups initially to estimate the effect size and to allow appropriate power calculations to be performed.

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 carrying mutations specifically within their pancreatic cells, in genes that are mutated in human pancreatic cancer, will be used. We might also alter genes within 'normal' cells that can help drive cancer, for example, wound-healing cells, or we may induce inflammation in the pancreas, as this is a risk factor in humans. Some mice will receive a transplant of tumour tissue or cells from mouse or human cancer (occasionally via surgery).

Mice will develop pancreatic or bile duct tumours or metastases (spread in other organs) that mimic the human disease. Pancreatic cancer is a very complex cancer involving many different cell types, so more refined models in cells or in less sentient animals don't mimic the disease well. Further, to be able to model mechanisms of metastasis to other organs, we need to use mice. Mice with early stage pancreatic cancer do not actually exhibit signs of suffering or distress, and where possible mice will be humanely culled before showing any signs of suffering, however, mice with late-stage pancreatic cancer will exhibit some clinical signs (usually a slightly swollen belly and loss of body condition around the haunches), and unfortunately, it is necessary to allow some mice to develop established pancreatic tumours to accurately model the clinical situation. Mice showing these signs will be humanely culled.

In some cases, to mimic clinical trials, a biopsy will be taken and then mice enrolled onto a mouse 'clinical trial', using either clinically relevant treatments, or further genetic alterations where drugs do not yet exist, combined with imaging. Where any surgery is performed some pain or discomfort is expected so this will be kept to a minimum with analgesia. We expect mice to recover normally in 2-3 days. Once on therapy, mice will be given treatment by experienced staff using the most refined techniques (single use needles and aseptic techniques).

For surgery or for imaging, anaesthesia will be used. The body temperature of the mice under anaesthetic will be maintained using heated platforms, and normal mouse food may be supplemented with gel diet or other treats after anaesthesia.

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

Pancreatic cancer is a complex cancer that involves several different cell types e.g., cancer cells, immune cells, wound-healing cells, and a particularly dense matrix, all of which play a major role in tumour development, spread to other organs, and resistance to therapy. Mouse models of cancer are widely accepted to be the most closely

representative of human cancers. Tumours progress through the same stages of pre-cancer as in humans and spread to the same organs. Other models cannot reproduce this situation, indeed, less sentient species like flies do not possess a pancreas. Terminally anaesthetised animals are not suitable for testing therapies.

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

Robust standard operating procedures for animal care, monitoring and minimal handling are in place. Social, environmental and behavioural enrichment are also provided. Laboratory staff will ensure that all animals receive the highest standard of care. We have a vast amount of experience with mouse models of pancreatic cancer, and close monitoring of tumour development allows us to recognize clinical signs before the animals exhibit signs of pain. Cage labels for identifying and monitoring external tumours and clinical signs associated with ageing are in place.

Most mice will not experience any clinical signs, because they will not express all the right gene mutations to develop pancreatic cancer. In some cases, mice will be humanely killed at early timepoints before displaying any clinical signs. Mice with early stage pancreatic cancer also do not exhibit signs of suffering or distress, and where possible mice will be humanely culled before showing any clinical signs.

Imaging, using small animal ultrasound, allows us to detect internal tumours at much earlier stages, so in many cases we can monitor effects of chemotherapeutic drugs in tumours before mice develop clinical signs. Where possible, trials of these drugs will be stopped early, for example, when a trial is producing negative data. Where required, for example pre- and post-surgery, preventative medicine (anaesthesia, pain relief, heated cages) will be used.

We have recently acquired equipment to facilitate ultrasound guided injection and will use this as a refinement to avoid laparotomy where possible.

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

We follow the Workman "Guidelines for the welfare and use of animals in cancer research", as well as local guidelines, to ensure we use best practice with all our tumour models. We will follow the LASA guiding principles for preparing for and undertaking aseptic surgery. We will also adhere to the ARRIVE guidelines (https://www.nc3rs.org.uk/arrive-guidelines).

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

We will engage with the AWERB culture of care and local animal users' group meetings to learn about advances, as well as consulting the NC3Rs guidelines. In our facility, compulsory user forums are held every 2 to 3 months for all personal licence holders, and 3Rs advances are introduced here and implemented across the facility.

63. Nutrition of poultry

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

Nutrition, Efficiency, Innovation, Gut health, Sustainability

Animal types	Life stages
Domestic fowl (Gallus gallus domesticus)	Juvenile, Adult, Aged animal
Turkeys (Meleagris gallopavo domesticus	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 develop nutritional strategies that enhance poultry performance and gut health while addressing critical challenges such as resource competition and the environmental impact of animal production.

By exploring alternative feedstuffs to replace soya, the project contributes to global sustainability efforts. This includes reducing deforestation and habitat loss caused by soya cultivation, lowering greenhouse gas emissions by minimising transportation and intensive farming, and supporting circular economies through the use of locally sourced agricultural by-products. These innovations aim to build more resilient and sustainable food systems while mitigating the environmental challenges of modern agriculture.

Additionally, the project employs disease challenge models to investigate pathogen behaviour and resilience. This research aims to identify nutritional factors that enhance disease resistance and inform effective management practices, thereby reducing economic losses. Ultimately, the findings will support the poultry industry in producing healthier, more robust flocks while aligning with sustainability and welfare goals.

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 to optimising poultry feed formulations, which can significantly enhance productivity, reduce costs, and promote sustainability in the poultry industry. The findings will:

Improve nutrient utilization and align feed precisely with birds' dietary needs, supporting better health and productivity.

Lower feed costs, which comprise 70–80% of poultry production expenses, by improving the understanding and use of conventional and alternative feedstuffs.

Reduce environmental impact by minimising nutrient excretion and enabling the use of lowerspecification diets without compromising performance or welfare.

Enhance poultry health through strategic feed additive use and balanced nutrition.

Support food safety by reducing gut contamination and the carriage of foodborne pathogens, contributing to the production of safe, nutritious poultry products.

Ultimately, this project addresses critical economic, environmental, nutritional, and global health challenges, serving as a vital step toward sustainable poultry production while benefiting researchers, industry stakeholders, and global food security.

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

The project outputs include a range of data from studies conducted under various protocols, such as production metrics (weight gain, feed conversion efficiency, egg production), microbiological and biochemical data, and insights into histomorphology and haematological parameters. Studies on nutrient utilisation and metabolism, including digestibility data, help refine feed formulations and optimise nutrient absorption. Protocols also generate information on the effectiveness of non-medicinal feed additives in preventing diseases, e.g., coccidiosis and Campylobacter, promoting gut health and productivity. Additionally, findings from precision feeding studies and large-scale trials contribute to refining dietary strategies, reducing animal use, and ensuring that dietary treatments are effective under commercial conditions while complying with regulatory standards. At the end of the project, expected outputs include peer-reviewed publications, presentations to scientific audiences, and dissemination to stakeholders to share findings and practical applications.

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

The outputs from this project will benefit a wide range of stakeholders, with both short-term and longterm impacts:

Poultry Industry and Farmers: In the short term, these findings will help optimise feeding strategies and improve productivity, feed efficiency, and bird health. This will lead to cost savings by reducing feed wastage and improving nutrient utilisation. The long-term benefits include more resilient poultry farming practices, reduced dependency on antibiotic

treatments, and better management of disease challenges, contributing to sustainable poultry production.

Feed Manufacturers and Additive Companies: The research will provide valuable data on the efficacy of non-medicinal feed additives, including but not limited to bacteriophages, prebiotics, probiotics and synbiotics, helping companies develop targeted products that improve poultry health and performance. This can lead to the development of innovative additives that contribute to subclinical disease prevention and overall gut health improvement

Regulatory Bodies (e.g., Food Standards Agency, Defra, Food Standards Scotland): The outputs will inform regulatory policies on the use of feed additives and disease control strategies, particularly with regard to food safety and antimicrobial resistance. By demonstrating safe and effective use of non-medicinal additives, this research may support regulatory approval for new products and practices.

Researchers and Academic Institutions: The findings will advance scientific understanding of nutrient utilisation, disease prevention, and the impact of feed additives on poultry health, fostering further research in the field of poultry nutrition and disease management.

Public Health and Food Safety Authorities: The long-term benefits include improved food safety and reduced transmission of foodborne pathogens like Campylobacter and Salmonella. By minimising microbial contamination and enhancing gut health, these findings will help reduce the public health risks associated with poultry production.

Consumers: Over time, the adoption of these strategies will lead to the production of healthier, safer, and more sustainably produced poultry products, directly benefiting consumers through improved food quality and safety.

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

This project brings together academics, poultry producers, and feed additive companies in collaborative research. By combining expertise in poultry nutrition, microbiology, metagenomics, molecular biology, and feed production, the project ensures its findings can be applied practically across the industry. Results will be shared through national and international conferences, workshops, and seminars, reaching key stakeholders like farmers, feed manufacturers, and food safety organizations. The research will also be published in peer-reviewed journals, highlighting both successful and unsuccessful approaches to expand the understanding of poultry nutrition, welfare, and gut health.

Species and numbers of animals expected to be used

Domestic fowl (Gallus gallus domesticus): 66,370

Other birds:

Turkeys (Meleagris gallopavo domesticus: 5300

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.

Poultry are used because the purpose of the project is to produce information that will be of value to the poultry production sectors (producers, feed additive manufacturers, feed manufacturers and poultry processors, etc.). For the type of work to be conducted there is no suitable alternative animal. The studies will look at birds at different stages of life—meat birds from hatch to slaughter, laying hens from maturity to their first egg cycle, and turkeys from hatch to slaughter. This approach ensures the findings are practical for real-world farming. The research also mimics commercial farming conditions by using smaller or larger groups of birds and includes challenges like food restriction and disease exposure. The goal is to improve feeding strategies and health treatments for poultry in the industry.

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

In this project, birds will undergo various procedures to meet the research objectives. They will be housed in cages or pens, either individually or in groups, and assigned to different nutritional treatments, which may include nutrient imbalances or meeting basic maintenance needs. Some birds may experience feed withdrawal up to 24 hours to mimic commercial farming practices, but in specific cases, it may be extended to 48 hours solely for precision feeding procedures. Additionally, test products such as probiotics, prebiotics, enzymes, or plant extracts may be administered through feed, water, litter, or by oral gavage or inoculation. Some birds will be exposed to sub-clinical disease challenges using pathogens like Eimeria, Campylobacter, or Salmonella, through methods like oral gavage, feed, water, or litter. Vaccination may also be used to pre-dispose birds to certain conditions. Blood samples may be taken to assess nutritional or health indicators, but no more than 10% of the bird’s circulating blood volume will be collected at once, with repeat samples limited to 1% per day. Excreta may be collected for nutrient retention or test product analysis, and cloacal swabs may be taken to check for pathogens. Birds will be monitored for welfare indicators, including the presence of footpad and hock lesions when required. At the end of the study, birds will either be euthanised by a Schedule 1 method, another approved method, or re-homed (for layers). The duration of experiments will vary from a few days to several months, depending on the study, and some birds may be reused or kept alive for further study. The number and type of procedures performed will depend on the specific protocol, with regular monitoring and interventions as necessary.

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

In this project, certain feeding studies will involve birds being provided with diets that do not meet their nutritional requirements, which may result in 15% body weight loss compared to age-matched control groups. In precision feeding procedures, any bird that shows signs of resistance will have the feeding procedure immediately halted, and any remaining feed will be recorded and discarded.

For laying hens, if feather pecking becomes an issue, beak trimming will be considered, provided it has not been done already or has been poorly performed. Veterinary consultation will be sought if any bird displays gentle pecking injuries. To prevent further pecking, affected birds will be treated with anti-peck spray. In cases of aggressive feather pecking, where forceful pecking leads to bleeding, appropriate measures will be taken to minimise suffering, and if recovery is not anticipated within 24-48 hours, the affected bird will be culled. If two or more birds, or 10% of the birds in a group, are culled or die due to injurious pecking, the entire replicate will be culled.

Any bird that develops severe swelling, ulcers, or scabs on footpads or hock lesions will be humanely euthanised. In cases of blood sampling, if a bird develops bruising or haematoma of the wing vein and the bird's welfare is compromised as a consequence (i.e., bird showing pain or distress, excessive vocalisation, reluctant to move, lethargic, etc.) the bird will be humanely culled.

To establish standardised and true digestibility of nitrogen, amino acids, and minerals, some birds will be provided with diets containing very little or no protein or minerals for a period not exceeding 7 days. Any bird that experiences more than 17% weight loss compared to its starting weight will be humanely culled.

If, during the pathogen exposure (e.g., Campylobacter spp., Salmonella spp.), clinical disease symptoms become severe or affect more than 30% of the birds in a pen, the entire pen will be culled to prevent suffering.

At the conclusion of the experiments, some birds will be euthanised using humane methods. For studies where euthanasia is not required, the birds may be rehomed to backyard flocks.

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)?

Although most of the protocols are classified as moderate, due to the close monitoring of the birds, it is unlikely that the procedures will exceed the mild classification. In laying hens, additional enrichment items such as ropes/strings will be placed within the cages (whenever possible) to encourage and promote normal behaviours.

What will happen to animals used in this project?

- Killed
- Rehomed
- 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?

While *in vitro* techniques and chemical assessments provide valuable insights into fundamental mechanisms, they cannot fully replicate the complexity of live birds in terms of nutrient uptake, gut health, and diet interactions. These studies are used extensively to refine hypotheses and experimental designs, thereby reducing the number of birds required by identifying non-functional treatments or optimal doses for in vivo trials. However, live birds remain essential to achieve the project's objectives, as they allow for the comprehensive evaluation of how dietary interventions affect overall health, nutrient absorption, and performance, which cannot be accurately modelled through *in vitro* methods alone.

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

Research scientists from our group have successfully used culture-based methods, both batch and continuous (e.g., bioreactor) and mathematical modelling. Through culture-based approaches, they have quantified bacterial populations and the rate of change of their metabolites, and explored microbiota dynamics under controlled gut-like conditions.

Additionally, mathematical modelling has been employed to simulate bacterial growth and predict microbiota dynamics, enabling the evaluation of interventions such as probiotics and prebiotics efficiently and effectively.

Why were they not suitable?

While culture-based methods and mathematical modelling provide valuable insights into gut microbiota dynamics and interactions, they are not suitable to entirely replace *in vivo* studies due to inherent limitations. Culture-based methods, despite mimicking gut-like conditions, often fail to replicate the full complexity of the gastrointestinal environment, including interactions between the host, microbiota, and immune system. Similarly, mathematical modelling relies on assumptions and simplified systems, which may not fully capture the intricate and dynamic processes occurring in a living organism.

In vivo studies remain essential to assess the whole-body response to interventions, such as, but not limited to nutrient uptake, immune responses, and long-term impacts on health, which cannot be accurately replicated by *in vitro* or *in silico* approaches alone.

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?

Due to the several commercial and Innovate UK projects (on the go and in the pipeline), we anticipate a need for a significantly larger number of birds in upcoming trials over the next 5 years. The reduced number of birds used in past studies was a direct result of closure during the COVID-19 pandemic and temporary budget cuts thereafter, which slowed down research activities. With these constraints now lifted and the increasing scope of our projects, we are expecting to undertake more extensive trials to meet the growing demands of these initiatives over the next five years.

Protocol 1 involves layers, broilers, and turkeys, with total bird numbers reaching 44260. (We anticipate running 1 layer, 3 broilers,and 1 turkey trial/year)

Protocol 2 focuses on broilers and cockerels, with up to 900 birds to be used in total. (We anticipate running 3 trials in total over the next five years)

Protocol 3 includes broilers and turkeys, with a total bird usage of approximately 6,500 (We anticipate running 4 trials /year)

Protocol 4 involves broilers and turkeys, with around 5,010 birds included. (We anticipate running 3 trials/year)

Protocol 5 also focuses on broilers, turkeys, and layers, with total bird numbers reaching 15,000 (We anticipate using 3 to 4 trials /year)

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 birds used in the project, we need to reduce the variation in the population of the hosts and standardise the conditions under which the experiments are performed as much as possible. To that effect, experiments will be planned well in advance, and birds with similar age and body weight (as much as possible) will be used for the individual experiments. The number of replications and birds per replication is determined based on previous experience, statistical analyses and power calculations. Each experiment is individually set up to maximise the ability to test for treatment effects combined with every effort to use the minimum number of birds. Experimental protocols are reviewed before each experiment to ensure that birds are not used unnecessarily. In addition, there are monitoring exercises after each experiment to see what lessons are learned. The 3Rs is part of the monitoring exercises. Part of the review of the experiment includes the input of expert statisticians.

Sample size calculations will be based on the magnitude of the effect that the study is designed to test, its variability, an adequate statistical power and the confidence level as required by European Food Standard Authority (EFSA) for assessment of the efficacy of feed additives or safety of feed additives for target species.

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

To optimise the number of birds used in this project, several measures will be implemented beyond good experimental design:

Pilot Studies: Small-scale pilot studies will be conducted to refine methodologies, identify effective treatments, and determine appropriate sample sizes before scaling up to full trials.

I***n Vitro and In Silico Approaches***: Culture-based methods and mathematical modelling will be used to screen hypotheses, assess preliminary outcomes, and narrow down variables, reducing the need for larger in vivo studies.

Efficient Use of Birds: Birds will be used to maximise data collection per individual or in bulk, with multiple measurements or endpoints being taken from the same individuals or groups where possible, to enhance statistical power and precision.

Statistical Tools: Advanced statistical techniques, such as power analysis, will be employed to ensure that the minimum number of birds required for reliable results are used in each experiment.

By implementing these measures, the project will adhere to the 3Rs principle, particularly reduction, while ensuring robust and meaningful outcomes.

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, poultry (chickens and turkeys) are primarily used as the animal model due to their commercial significance and well-characterised digestive physiology, which makes them ideal for research in feed formulations and nutrient digestibility. These species provide highly relevant data for the poultry production industry.

With over 20 years of experience using these animal models and experimental methods, we have consistently refined our protocols to minimise animal discomfort. For instance, our improved gavage technique, which replaces a 5ml syringe (with 3-4 cm of clear vinyl tubing) with a 1ml sterile insulin syringe, makes the procedure faster and less stressful for the birds. When transitioning birds from floor pens to metabolic cages, we acclimate them with a 50:50 diet of their previous standard commercial diet and the experimental diet, helping to reduce stress. In digestibility studies, the housing period in metabolic cages is kept as short as possible (up to 7 days), and birds are provided with ad libitum access to food and water throughout the experiment to minimise discomfort.

For laying hens, we use nylon ropes as an environmental enrichment tool, allowing the birds to engage in natural behaviours such as pecking, perching, and stretching, which promotes well-being and reduces stress. By continuously refining our methods and incorporating enrichments, we ensure that the birds experience the least amount of pain, suffering, distress, or lasting harm during the study.

After each study, a wash-up meeting is conducted to review the procedures, assess the effectiveness of the methods, and identify areas for improvement. These meetings help us address challenges and suggest refinements for future studies, enhancing animal welfare and experimental outcomes. This ongoing review process ensures that each study benefits from the lessons learned in previous ones, optimising protocols and minimising animal distress in subsequent research.

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

The selection of animals for research depends on their biological relevance to the study's objectives. For this project, poultry are the most appropriate model because they are the target species for the research, and their physiology, nutrition, and health outcomes directly inform industry practices. Using animals with less sentience, would not provide the necessary data due to significant differences in anatomy, metabolism, and immune function.

Additionally, all research involving animals is designed to adhere to the 3Rs principle. We use poultry only when there are no valid alternatives to achieve the scientific objectives, and we work diligently to minimise their numbers and refine our practices to ensure the highest standards of welfare.

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

The birds in this project will be monitored daily by trained staff (e.g., NACWO, Project manager and NVS) to ensure their well-being. Specialist avian veterinary support is

available on-site to provide additional oversight. For disease challenge studies, only sub-clinical infection models are employed. All studies are rigorously assessed by the institute’s Animal Welfare and Ethical Review Body (AWERB). AWERB plays a critical role in evaluating the ethical and scientific validity of proposed studies. Poststudy review meetings further enable the continuous refinement of our procedures.

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

The PREPARE and ARRIVE guidelines will be followed.

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 them in our work, we will regularly be checking information on the NC3Rs website. Information on advances in the 3Rs in this field is communicated through our institute's AWERB. Information on advances in methodologies is often communicated through scientific publications and at conferences, and we will stay informed about new developments via these channels.

64. Autonomic control of cardiac function and rhythm

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

Sudden cardiac death, Cardiovascular disease, Arrhythmias, Heart failure, Autonomic nervous system

Animal types	Life stages
Rabbits	Adult
Guinea pigs	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 programme of work is to investigate how the nervous system affects key processes in heart function and to gain an understanding of the relationship between dysfunction of this system and lethal abnormal heart rhythms (ventricular arrhythmias) which can lead to Sudden Cardiac Death.

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?

Sudden Cardiac Death is a major unsolved clinical problem claiming 100,000 lives in the UK each year. The majority of these deaths are due to lethal heart rhythm disturbances known as ventricular fibrillation (VF). The mechanisms of VF are still poorly understood and there is no effective treatment. Dysfunction of the autonomic nervous system (a network of nerves that extends throughout the body, including the head and spinal cord) is known to be a significant factor in the development of VF, however a better understanding of the underlying mechanisms is necessary.

The novel data obtained from our recent studies have advanced our understanding of the complex network of nerves in the heart and the development of irregular heartbeats in both healthy and diseased animal models. Despite these important insights, critical gaps in knowledge remain, and there are key questions that still need to be addressed.

This licence is essential to advance the understanding of why sudden cardiac death occurs and the role that the nervous system plays. The experiments are designed to identify key signalling pathways that will further scientific knowledge, which we hope will one day improve current therapies and develop new ones. The data collected will be combined with mathematical modelling to improve and advance what is currently available which will incorporate up to date information on structure and physiology of nerves which plug into the heart.

A new emphasis of this licence is the exploration of a new disease model which could potentially better represent patients with heart failure following a myocardial infarction (heart attack). We have also identified a gap in the understanding of the development and treatment of cardiovascular disease in females partly due to the limited representation of females in animal studies. Thus, we aim to gain insight into the differences between male and female cardiovascular physiology in a set of preliminary experiments. We will also conduct a more in-depth exploration of the nerve-heart interactions that become dysfunctional in diseased states.

- The current work is essential to advance the understanding of the mechanisms underlying autonomic modulation of ventricular fibrillation (VF) and cardiac function.

- The investigations may identify key signalling pathways that could lead to the development of new therapies for this lethal condition.

- Data will be published in academic journals that will be of interest to cardiac- and neuro-physiologists and clinical personnel with an interest in cardiac disease and the wider scientific community.

This licence will follow the same theme of the previous licence to facilitate the continuation of the research. Of course, new data and new findings are accumulated everyday which drives the direction of the ongoing research and thus this application. The remit of the work in this licence will still address the theme 'Autonomic control of cardiac function and rhythm' and will focus on molecular, cellular and whole heart models to investigate irregular heartbeats and Sudden Cardiac Death.

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

This licence is essential to advance the understanding of why sudden cardiac death occurs and the role that the nervous system plays. The experiments are designed to identify key signalling pathways that will further scientific knowledge, which we hope will one day improve current therapies and develop new ones.

A new emphasis of this PPL is the exploration of a new disease model which better represents patients with heart failure and the investigation into the differences between male and female cardiovascular physiology in a set of preliminary experiments. We will also further explore the nerve-heart interactions which are key to understanding the development of lethal heart rhythms and sudden cardiac death. These experiments and the others in this PPL will advance our understanding of cardiovascular physiology and how this becomes dysfunctional in disease states leading to an increased risk of life threatening arrhythmias. This will provide valuable insights to inform the development of clinical therapeutics.

The data collected will be combined with mathematical modelling to improve and advance what is currently available which will incorporate up to date information on structure and physiology of nerves which plug into the heart. We aim to gain a better understanding into cardiac nerve distribution and ion channel distributions and the subsequent regional effects on the heart.

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

Data produced throughout this project will be presented at national and international conferences and published in academic journals.

Short term: Gain an understanding of the mechanisms underlying the modulation of arrhythmia which will inform studies using the myocardial infarction (MI) models of heart failure and enable comparison with disease models. Studies in cardiac cells will help us to identify ion channels that are important in modulation of arrhythmia. This data will also be used to determine relevant mathematical models of heart function. Colleagues and collaborators will benefit from data that will inform their experiments and approaches.

Medium term: The mechanisms and pathways identified will enable us to test target specific blockers/activators with potential to aid development of therapies. All of the above can be investigated in MI models to gain understanding on how these mechanisms and pathways are modulated in disease states. This data will also be used to determine relevant mathematical models of heart function. Colleagues and collaborators will benefit from data that will inform their experiments and approaches.

Long term: Advance the understanding of the mechanisms underlying ventricular fibrillation (VF) and cardiac function. The investigations may identify key signalling pathways that could lead to improvements in current therapies and the development of new therapeutic modalities for this lethal condition. Data will be published in academic journals that will be of interest to cardiac- and neuro- physiologists and clinical personal with an interest in cardiac disease and the wider scientific community.

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

We will maximise the outputs of this group through collaborations with other departments within the university and with other universities. We will disseminate new knowledge at national and international academic meetings and conferences and through publication of our findings in scientific journals.

Species and numbers of animals expected to be used

- Guinea pigs: 250
- Rabbits: 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.

It is impossible to investigate modulation of ventricular fibrillation (VF) in humans due to its lethal nature. The precise nature as to how neural and cardiac remodelling and their interaction in MI leads to increased incidence of VF cannot be studied in a controlled manner in patients as experimentation would require repeated episodes of lethal arrhythmias to explore the effects of nerve effects. Hence animal models are essential for scientific advancement relating to understanding the mechanisms involved. The use of whole animals and in vivo experiments are not ideal for studying pure effects from direct nerve stimulation on the heart due to substances in the blood and brain mediated reflexes that are difficult to control. Mathematical modelling is imperfect and still being developed to a point that would be acceptable to replace procedures requiring animals.

Hence we use the most refined model for the intended purpose of investigating the autonomic nervous system (ANS) as it allows tightly controlled studies without the confounding influences. Amongst small experimental animals, rabbits have the most comparable cardiac electrophysiological and ion channel characteristics to humans. Guinea pig cardiac electrophysiology is more comparable to humans than rats and mice and they have different expression of some ion channels (potassium channels) compared to rabbits, which play an important role in arrhythmia development.

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

Heart preparations will be prepared and removed during non-recovery anaesthesia for animals entering protocol 1. This will involve subcutaneous and intravenous injections, tracheotomy, exposure of tissues of interest and isolation and resection of vasculature / muscle (optional i.e. for innervated heart preparation), all of which will occur under terminal anaesthesia. The heart and associated tissues will be removed immediately after intravenous overdose of anaesthetic. Animals in protocol 1 may also have optional blood sampling under anaesthesia. Amongst small experimental animals, rabbits have the most comparable cardiac electrophysiological and ion channel characteristics to humans. The physiology of the Guinea pig action potential is different to rabbits and is more influenced by the potassium current known as IKs which plays an important role in the development of arrhythmias which is important to investigate.

Animals from protocol 2 will have general anaesthesia and surgical procedures performed to permanently tie or temporary occlude a coronary artery (or not for Sham animals) to produce a myocardial infarct. This will involve subcutaneous and intravenous injections, optional blood sampling under anaesthesia, thoracotomy and exposure of tissue of interest, 1 or more coronary arteries will be ligated or temporarily occluded to cause an area of ischemia and infarct for a MI (Sham animals will not have a coronary tie) and administration of intrathoracic steroids. Surgical subcutaneous implantation of a telemetry device is also optional. Animals can also have optional imaging or experimental measurements taken under recovery anaesthesia during the post operational recovery period. In humans, heart attacks can sometimes go undetected. When left untreated, they can increase the risk of arrhythmias and sudden cardiac death due to progressive structural and electrical remodelling of the heart. To better understand and accurately reflect these changes that occur over time, we will take measurements from the heart preparations at various recovery time points, extending up to 16 weeks. After the recovery

period the animals will be prepared for terminal experiments which will involve subcutaneous and intravenous injections, optional blood sampling, tracheotomy to expose of tissues of interest and isolation and resection of vasculature / muscle (optional i.e. for innervated heart preparation), all of which will occur under terminal anaesthesia. The heart and associated tissues will be removed immediately after intravenous overdose of anaesthetic. Animals under this protocol can have optional administration of dietary supplements in food or water, for example Beetroot juice for investigations into how these supplements can protect against heart failure or arrhythmia development.

Although pathological signs of heart failure are evident post-mortem, these animals do not exhibit the typical clinical symptoms of heart failure. From two days post-surgery, they resume normal eating habits, show no signs of weight loss, and actively engage in daily activities. Pain relief is provided pre and post operatively and extensive monitoring of animal welfare occurs.

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

To develop the heart failure model we perform an open-chest surgical procedure that is rated at moderate severity. Premature death is the most significant adverse effect that we try to keep to a minimum through sound aseptic surgical techniques and rigorous post-op monitoring with rapid reactive treatments if symptoms develop. Rabbits do not commonly display symptoms of heart failure like humans, but the most common adverse harm is pain, which is controlled with regularly monitored and reviewed good pain medication. At the end of procedure, animals are deeply anaesthetised and the heart is removed for use in the laboratory for scientific study.

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)?

500 Rabbits - Moderate (50% of total rabbits)

500 Rabbits – Non-recovery (50% of total rabbits)

250 Guinea pigs – Non-recovery (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?

It is impossible to investigate modulation of VF in humans due to its lethal nature. To get a better understanding of the mechanisms involved in the initiation and development of arrhythmia and SCD whole heart models are essential to understand the spread of electrical activity and thus the regional electrophysiology. Cellular experiments can then investigate deeper into the specific ion channels involved and their heterogenous

distributions in the heart. Hence animal models are essential to advance our understanding of the mechanisms involved. This data will inform the development of relevant mathematical models which will hopefully allow for some replacement in the future.

Currently there are no non-animal alternatives and no appropriate mathematical models available.

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

Mathematical modelling has provided some useful tools to investigate some aspects of cardiac physiology.

Why were they not suitable?

Mathematical models are in development, incomplete and are currently not suitable; as they do not fully address complex cardiac rhythms owing to the lack of a comprehensive understanding of all the processes involved.

To note: we had a 5-year programme grant in which we used our data to incorporate into current mathematical models and we will be continuing this work in future grants. Through continued development this will hopefully allow some replacement.

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?

Group size calculation is informed by expected differences in parameters based on our previous data (e.g. heart rate, action potential duration, ventricular fibrillation threshold) to provide adequate statistical power.

These are calculated based on the standard deviation (SD) of the data and estimated detectable change (DC) of the parameters studied. The least number of experiments required to demonstrate a scientific effect are used. Experiments are carefully designed and performed under tight control conditions and analysed in acceptable small cohorts, so statistical differences are analysed as work progresses and discovered early, so unnecessary additional experiments can be stopped

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

- We perform experiments in-vitro as confounding effects of circulating hormones/autonomic reflexes in in vivo setting would increase variance

- This allows our experiments to be tightly controlled from a homogenous population of animals, so the smallest number is used to achieve the objectives

- Statistical analysis is discussed with our departmental statistician when needed

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

Use of good laboratory practice (GLP)

Rigorous experimental data appraisal to ensure quality and low failure rates

Sharing of tissue e.g. sharing of muscle tissue with collaborators

Covering more than one objective for each 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.

Protocol 1

Amongst small experimental animals, rats and mice have a different action potential morphology to humans due to differences in calcium handling in the cardiac cells. Rabbits have the most comparable cardiac electrophysiological and ion channel characteristics to humans. The physiology of the Guinea pig action potential is different to rabbits and is more influenced by the potassium current known as IKs which plays an important role in the development of arrhythmias. This is the most refined model for the intended purpose of investigating of the autonomic nervous system (ANS) as it allows tightly control studies without confounding influences.

For isolated heart, single cell, molecular biology and immunohistochemistry experiments, the isolated heart is the most refined method for these intended purposes. The preparation is harvested during non-recoverable general anaesthesia following a sub-cutaneous injection of sedatives, which is the least painful injection route which has been refined from previous use of intramuscular routes.

Previously, animals were ventilated with room air but we have since refined this method with use of an oxygen concentrator which helps to maintain the rabbits oxygen saturation levels. We now also use continuous propofol infusion as oppose to boluses to allow better control of anaesthesia.

Protocol 2

Inducing myocardial infarction (MI) has clear adverse effects on the animal. The procedure involves a thoracotomy and the induction of a myocardial injury, which results in pain and other complications such as ventricular failure, thromboemboli and death. Experience with this model indicates that the expected harm can be controlled and minimised with resultant pathophysiology that can be used with human relatable data.

Refinements related to the MI model

- Use of rabbits 2.5-3kg to reduce mortality rate seen previously in lighter rabbits
- Refining anaesthetic protocols to ensure calm induction for the animals
- Anaesthetise vocal cords with local anaesthetic before endotracheal tube (ET) tube insertion
- Steroids used prior to closing thorax to reduce adhesions
- Optimisation of ligation to produce an infarct percentage that is sufficient enough to produce the required scientific outcomes but refined to a point that causes less severe outcomes for the animals

- Reduction in mortality rate from <30%. With refinement and control of procedures, our more recent data over the past 5 years would support a mortality rate of 0-5% for the MI/Sham models, all of which occurred under anaesthesia. See table below for more details:

Year	Mortality of MI/Shams	Mortatlity %
2020	1 out of 25	4.0
2021	1 out of 19	5.3
2022	0 out of 25	0.0
2023	1 out of 23	4.3
2024	1 out of 31	3.2

Average 3.4%

It is important to note that this procedure has been very well refined due to the many years of experience of those performing the procedure. However, for changes to the model/ procedure or during the training of new surgeons, there may be an increase in mortality for a short time until the new model/ procedure is optimised or until the new surgeon has become proficient in the technique. We do not anticipate the mortality rate to ever go above 10% in these circumstances.

- Use of effective analgesic regimen
- Frequent monitoring including pain scoring
- Reduction in the need to transport animals to different sites as all experiments are performed in the same building that the animals are housed in. We have also minimised transport of rabbits within the building and where possible, rabbits are sedated before being removed from the rabbit room.

- Addition of the temporary ligation model which will reduce damage to the heart and associated adverse effects as well as being a more relevant clinical model with the potential to replace other models in the future

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

Rabbits are preferred as the structure and physiology of the heart is more similar to humans, than rats or mice. Rats and mice have a different action potential morphology to humans due to differences in calcium handling in the cardiac cells. We use Guinea pigs because the physiology of the Guinea pig heart is different to rabbits and is more influenced by potassium ion channel currents (e.g. IKs) which plays an important role in the development of arrhythmias.

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

- Use procedures designed to cause the least pain, suffering or lasting harm

- Transport stress is minimised by acclimatising animals to handling and animals are box trained prior to delivery and assessed as fit to travel before transport.

- Reduction in the need to transport animals to different sites as all experiments are performed in the same building that the animals are housed in. We have also minimised transport of rabbits within the building and where possible, rabbits are sedated before being removed from the rabbit room.

- Pre-emptive, intra- and post-operative analgesia, antibiotics, and antiarrhythmic drugs under aseptic conditions used wherever possible

- Best practice post-operative monitoring of animals and rapid intervention in cases of postoperative complications

- Use the Rabbit Grimace Scale (Keating SC et al, 2012. PlusONE 7(9).e44437 and Body Condition Score

- Continued collaboration on the MI-model

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

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

Attending conferences such as NC3Rs and scientific conferences

Keeping up to date with relevant literature relating to 3Rs advances and discuss advances at our group meetings

Consider adopting any advances that will refine techniques based on the latest science and technologies

Periodically review new 3Rs information, experimental data and success and failure rate of procedures.

Ensure minimal number of animals is used for each project and discontinue animal use for projects that are not showing promising data

Design animal experiments that are robust and reproducible and ensure thorough analysis and regular review of data

Use animal data to address important scientific questions and develop appropriate mathematical models of the heart to reduce the need for animal experiments in the future

65. Understanding and overcoming mechanisms of therapy resistance in blood cancers

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

Blood Cancer, Therapy, Microenvironment, Drug resistance

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 principal aim of the proposed project is to improve our understanding of how cancer cells grow and spread with particular focus on the role of the tumour microenvironment and how this contributes to therapy resistance. By gaining deeper insights into these mechanisms, we then aim to devise innovative and targeted therapeutic approaches to effectively combat therapy resistance, test them in relevant models and generate data to support future 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?

Therapy resistance is a clinical challenge shared by the four blood cancers which form the focus of this project: acute myeloid leukaemia (AML), chronic lymphocytic leukaemia (CLL), diffuse large B-cell lymphoma (DLBCL) and multiple myeloma (MM). Importantly, the dynamic interplay between cancer cells in these diseases and their microenvironment is thought to substantially contribute to therapy resistance. The tumour microenvironment

consists of a variety of immune cells, such as T-cells, macrophages, and dendritic cells, as well as structural cells, including fibroblasts, endothelial cells, and extracellular matrix components, all of which play crucial roles in influencing cancer cell behaviour and response to treatment. Modelling the complex multicellular 3D microenvironment for these diseases *in vitro* is challenging and often lacks physiological fidelity. Therefore, *in vivo* models are crucial to gaining a greater understanding of this complex relationship to allow for the development and testing of novel, rationally targeted, therapeutic strategies to overcome microenvironment induced therapy resistance. Importantly, this work will provide critical *in vivo* data for novel compounds tested, a critical step in the drug development pipeline prior to clinical trials.

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

A significant output from this project will be a greater understanding of the mechanisms by which blood cancers interact with and alter their microenvironment, which creates a protective niche capable of driving therapy resistance. Specifically, whether there is a convergence on specific cell signalling pathways or whether the genetic, transcriptomic and phenotypic diversity of these diseases results in equally divergent changes in their microenvironment. This new information will be published in peer reviewed scientific journals (e.g. Blood, Haematologica, Hemasphere) and presented at both national and international conferences (e.g. European hematology association congress).

Furthermore, the project will test a series of promising therapeutic compounds being developed within our lab, and with collaborative groups, that target microenvironment driven therapy resistance. These will be tested both alone, and when indicated by *in vitro* assays, in combination with current standard of care therapies. The murine models described in this application will form a vital component of the pre-clinical assessment of these novel agents, which may lead to their evaluation in subsequent clinical trials in patients diagnosed with these diseases.

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

In the short-term (3-5 years), the pursuit of this project promises to yield knowledge that will be of benefit to the scientific community, particularly those within the cancer field. Additionally, the testing of compounds designed to disrupt cancer-supportive interactions within the tumour microenvironment may provide vital proof of principle for these novel compounds, that will be of benefit to the

pharmaceutical sector. Crucially, in the longer term (5-10 years), this groundwork will lay the foundation for future therapies addressing the clinical unmet need of patients with refractory disease stemming from pervasive therapy resistance.

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

To maximise the reach of the outputs from the proposed project, dissemination of new knowledge will be made in open-source academic journals and pre-prints made freely available on services such as bioRxiv. Additionally, new knowledge will be presented at both national and international scientific conferences. This dissemination of information will take place irrespective of whether positive or negative data is obtained and will include any novel experimental tools or approaches developed.

We will also directly inform patients of our research outputs in an accessible manner through our annual “Patient open days”, where we host patients and their relatives/carers. Additionally, we will strive to share our outputs with relevant charities and the public by taking part in events such as “Science Café” events run by a local cancer charity where

researchers are asked to discuss their research with the public. Similarly, we will leverage a newly established cancer centre at our establishment to increase our public engagement and outreach.

Importantly, I am embedded within a collaborative group of blood cancer researchers which has undertaken some of the in vitro testing of the novel compounds within this proposal. Therefore, these groups will stand to benefit from the work described in this proposal by advancing their research into the necessary in vivo testing that is required to validate novel therapeutic strategies. Additionally, I will seek to collaborate externally to share resources and knowledge gained throughout the project. These efforts will involve collecting several tissues from mice used in our studies to generate a tissue bank which will be made available to other researchers.

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.

Species: Mice are used because they are the lowest order vertebrate that share sufficient relatedness to humans and allow us to generate the humanised immune microenvironment that is critical to our aims.

Strains: Immunocompromised mice are used because their genetic alterations allow for the implantation of tumours without rejection, thus reducing the number of animals required.

Ages:

Pregnant adults and neonates: We will be breeding immunocompromised mice for experiments.

Juvenile: The implantation of cells to generate a human-like immune system is more efficient in mice younger than 6 weeks of age.

Adults: It takes 10-12 weeks for the human-like immune system to develop prior to the implantation of tumour cells. Therefore, tumour implantation takes place when mice are adults.

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

Breeding

Animals will be bred and typically undergo ear notching for identification, with the tissue from this being used for genotyping. In rare instances, a second sample may be taken if the genotyping fails. This breeding will provide animals for our experiments.

Handling

Mice will be handled by the investigator prior to undergoing procedures to reduce stress.

Experiments testing candidate treatments for blood cancers

Conditioning

Animals may undergo conditioning to prepare them to be implanted with donor cells. This involves the administration of a conditioning agent (typically a chemotherapy) by intraperitoneal injection.

Developing a human immune system (humanisation)

Shortly after, they may be implanted with healthy human haematopoietic stem cells to enable them to generate a human immune system. This will be done by injecting the cells in as small a volume as possible into the tail vein. The human immune system will develop over the course of several weeks and will be monitored weekly starting at 8 weeks after the injection of the stem cells by taking small volumes blood from the tail vein or the saphenous vein.

Once the human immune system has developed sufficiently (typically within 12 weeks of the stem cells being injected), mice will be further implanted with tumour cells either systemically or subcutaneously (under the skin) by one of two methods:

Systemically (into their circulation)

Systemic tumours will be induced by a single injection of tumour cells into the tail vein, again in as small a volume as possible. Subsequently, mice may have their tumour burden monitored through weekly blood samples taken from the tail vein or saphenous vein.

Subcutaneously (under the skin)

Implantation of tumour cells or tumour fragments under the skin of the flank of the mouse. The former would be done by injecting the cells under the skin either while manually restraining the mouse or under short general anaesthesia, while the latter would require the animal be anesthetised and the tumour fragments placed under the skin either using a trocar (a sharp hollow tube) or by making a small incision in the skin to create a pocket for the fragments. The mice will develop a solid tumour which will be regularly monitored for size using callipers.

Testing new treatments

In either case, when sufficient tumour burden is present, the mice will be administered regularly with novel candidate therapies or vehicle controls to test the effectiveness of new therapies at reducing the tumour burden. This may be done by regular intraperitoneal injection (abdominal cavity), intravenous injections (tail vein) or subcutaneous injections (under the skin) injection.

After the treatment phase is complete, mice will be killed and their tissues and tumours collected for analysis to determine the effectiveness of the tested treatment.

Maximum tolerated dose experiments

Some mice will not undergo implantation of tumours but may undergo conditioning and humanisation as described above, followed by administration of candidate treatments (via one of the routes described above) for up to 28 days and assessed for moderate adverse clinical signs to determine the tolerability of the candidate treatment.

Disease progression experiments

Some mice may undergo conditioning, humanisation and systemic tumour implantation but will be killed by at different time points to determine the disease course over time.

Effect of tumour cells on microenvironment experiments

After conditioning and humanisation some mice will be implanted with systemic tumours, while others will act as controls without tumours. Subsequently, mice will be killed and their bone marrow or lymphoid tissues analysed for tumour induced changes in comparison to those who did not receive tumour implantation.

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

Conditioning

We expect that animals may experience some weight loss which is typically recovered within 2 weeks. In rarer instances, animals may experience adverse effects due to the chemotherapy administered which may include gastro-intestinal distress, anaemia and nausea. However, we will minimise this risk by dividing the dose into multiple administrations (typically 2).

Humanisation

Humanisation by administering haematopoietic stem cells is typically well tolerated and we expect most animals not to show any adverse effects. A small number may show some redness of the skin which is indicative of graft versus host disease (GVHD) at which point they will be humanely killed.

Systemic tumour

We anticipate that for most animals the primary adverse effect of systemic tumour engraftment will be some weight loss. Some mice may show a greater loss of condition presenting with symptoms including a hunched posture, dehydration and piloerection. If these symptoms can’t be improved under guidance by the NVS within 24 hours, the animal will be humanely killed. Additionally, the animal will be immediately killed if rapid weight loss of 15% or more, unsteady gait, secondary tumours, laboured breathing, and failure to take in food or water over a 24-hour period manifest.

Subcutaneous tumour

We anticipate all mice will experience mild to moderate discomfort from the growth of a solid tumour to a maximum size of 1.5 cm. Also, in the event of infected ulceration of the tumour, the animal will be humanely killed. The same action will be taken if the tumour interferes with locomotion. Additionally, the metastasis of the tumour may cause similar adverse effects to those listed above for systemic tumours.

Treatment administration

Novel drugs/molecules will be administered to identify the maximum tolerated dose. From these experiments, we expect that only two mice per drug tested will show moderate clinical symptoms including weight loss, dehydration, a staring coat (piloerection), hunched posture and subdued behaviour. Animals showing any of these signs will be humanely killed. Importantly, this would have identified the highest safe dose to give during the testing of these drugs/molecules for the purpose of reducing tumour burden.

Consequently, we do not expect adverse effects to occur when treating tumour burdened mice with the selected dosage.

Blood sampling and injections

Mice will undergo repeated blood sampling and injections which may cause transient discomfort but may result in a cumulative suffering of moderate severity.

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 that 35% of mice will experience mild severity and that due to the cumulative effect of the procedures described above, we expect 65% of mice used in this project to 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?

To achieve the aims of this project, a physiological and dynamic model of the microenvironment in which blood cancers reside is necessary. Blood cancers rely on complex crosstalk within tissue microenvironments for survival, proliferation, and protection from the effects of anti-cancer therapy. These environments, such as the bone marrow (AML and MM) and lymphoid organs (CLL and DLBCL), are highly dynamic and consist of several cellular populations (such as stromal cells and immune cells e.g., T cells) making them challenging to model *in vitro*. We have generated data that demonstrates that these interactions contribute to treatment resistance. Even the most complex 3D *in vitro* models fail to mimic the full biological complexity of these environments. Thus, the integration of animal models becomes indispensable for our research, allowing us to closely mimic the intricate physiological conditions, cellular interactions, and dynamic responses that occur within these microenvironments.

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

Through our collaborative approach, we integrate in silico modelling and in-depth *in vitro* modelling into our pipeline, prior to proceeding with animal models. For example, we utilise computational modelling of microenvironment stimulation to identify candidate drug targets, which are subsequently tested in 2D co-cultures consisting of the cancer cells and cells mimicking the microenvironment stimuli that are present *in vivo*. Only the most promising therapeutic candidates will be taken forward into animal studies.

Additionally, we also make use of 2D co-cultures to look at the basic mechanisms of communication between cancer cells and stromal cells in their microenvironment. However, the insight gained from these studies requires validation *in vivo* due to the simplistic nature of these models in comparison to the complex architecture of these

environments *in vivo*. 3D co-culture models were also considered as they aim to bridge the gap between 2D co-culture models and *in vivo* experiments.

Why were they not suitable?

As mentioned, in silico modelling and 2D *in vitro* co-cultures are crucial components of our investigative pipeline prior to reaching the *in viv*o stage. However, data obtained from these models requires validation in a more complex and physiologically relevant model.

While computational modelling identifies promising therapeutic targets these models only capture interactions that we hypothesise to be important and therefore must be validated *in vitro* and *in vivo* to test whether computational predictions can be validated in the context of complex multicellular interactions with unexpected emergent behaviour.

Despite our 2D co-cultures reproducing some aspects of microenvironment stimuli, this is limited to a maximum of three stromal cell lines or a single cell line expressing a specific stimulatory molecule. In reality, within the bone marrow and lymphoid organs, cancer cells receive and send signals both directly and through the release of soluble molecules with various cell types simultaneously. Additionally, the 3D architecture of this environment is not reproduced in 2D cultures. While, 3D coculture models aim to fulfil this remit, they still fall short of accurately mimicking the complexity of the cancer microenvironment and their added technical complexity leads to issues with reproducibility. Therefore, the rational final step in our pipeline is the animal models described within this project.

Importantly, the testing of novel therapeutic compounds using *in vivo* models is a critical step in the preclinical development of future therapies. The systemic administration and pharmacokinetic properties of these compounds are not fully captured using current *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?

The minimum number of animals for each experiment required to test our hypothesis while ensuring enough power to achieve statistically and biologically meaningful results was determined using sample size calculations.

We will limit variation by using mice which are the same age at the start of the protocol (4-6 weeks) and from the same strain. Additionally, we will utilise balanced sex distribution with equal numbers of male and female mice across the treatment and control groups, ensuring observed effects are not biased by sex imbalance and remain robust and generalisable.

To mitigate for the fact that animals will undergo the procedures described in this protocol at different times, we will utilise a randomisation within blocks design. Every treatment and control group will be represented an equal number of times within each block. Animals (the experimental unit) will be randomly assigned to a treatment group or control group within each block. Once tissue is collected, and prior to analysis, it will be assigned a unique ID which will mask the control or treatment group it belongs to.

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

We used the experimental design guidance and the Experimental Design Assistant (EDA) provided by NC3Rs to plan our study including guidance on sample size calculations, randomisation and blinding.

Additionally, to ensure that our model system is reproducible we will first perform small scale studies of the disease progression, allowing us to refine our methods so that in the larger scale studies fewer animals will be required. Also, maximum tolerated dose testing will allow us to eliminate any candidate therapeutic that has undesirable adverse effects using a small number of animals and prior to progressing to larger cohorts used in the efficacy testing protocols.

Where possible we will test candidate therapeutics which share a vehicle in parallel so that the vehicle controls can be shared to reduce animal numbers.

We are also implementing a randomised block design to reduce variability and consequently allow us to reduce animal numbers.

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 developed a robust *in vitro* pipeline for drug development and testing, in collaboration with a systems biologist. This pipeline enables us to make *in silico* predictions about the most suitable therapeutic strategies, which are then tested *in vitro*. Only the most promising candidates progress to *in vivo* testing, thereby reducing the number of animals required.

To ensure the reproducibility of our model system, we will initially conduct small-scale studies on disease progression using our systemic model. This approach allows us to refine our methods, further reducing the number of animals needed in larger-scale studies. Additionally, maximum tolerated dose testing will help us identify and exclude candidate therapeutics with undesirable adverse effects early on, minimising animal numbers used. This prevents such candidates from advancing to larger cohorts in the efficacy testing protocols.

Data from testing our initial candidates will be used to update our sample size calculations, as previous data was unavailable and standardized effect sizes were initially used. At the end of the experiments, we will harvest as many tissues as possible per post-mortem, store them for future use (fixed and snap-frozen), and make them available to other researchers. This resource will be advertised through a newly established cancer research centre.

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 xenograft mouse models for our experiments, these are immunocompromised mice which will be implanted with tumour cell lines or patient derived tumour cells. The principal motivation for taking this approach is that it is the only method which would allow for us to model different tumour subtypes and test different therapeutic candidates designed to be particularly beneficial to specific subtypes of tumour. Consequently, the adverse effects and harm associated with these kinds of models are unavoidable. However, we have detailed steps to mitigate these harms as much as possible to ensure we obtain robust results without compromising the integrity of the experiments. An alternative approach would have been to use genetically modified mice which are susceptible to spontaneous tumours like Eμ-TCL1 mice. However, these mice develop mouse tumours in a solely murine microenvironment which does not faithfully recreate the human disease as well as the models we have elected to use. Furthermore, as these mice develop genetically similar tumours, they would not be a good representative model of the heterogeneity we see both between and within the four different blood cancers being used. Historically, they are also associated with increased risk of candidate therapies not translating well to human patients. Finally, and importantly, these models are often associated with chronic impact to the animal's health and often a shorten lifespan. Therefore, we feel that these models are not suitable and potentially inherently more harmful than the models we have elected to use.

Importantly, in some experiments the immunocompromised mice will first undergo humanisation to generate a human-like immune system and further increase the fidelity of this model for human disease. This is critical to our aims as we have shown that interactions of the cancer cells with the microenvironment are key to treatment responses. Of the two methods available to do this, we have chosen the most refined method which presents the least harm to the animals. We will introduce a human-like immune system using haematopoietic stem and progenitor cells (HSPCs: stem cells responsible for generating blood and immune cells). Using this method, means that the developing human immune system will be educated in the mice and so will not induce graft versus host disease.

The alternative would have been to use peripheral blood mononuclear cells (PBMCs) which consist of immune cells educated in the human body and so would recognise the mouse as foreign, which would initiate severe graft versus host disease within a few weeks. Another possible alternative would have been the use of syngeneic models which also allow for investigation of the tumour microenvironment, but this is solely a murine environment unlike our selected model. Additionally, the syngeneic model would limit our ability to model inter-patient heterogeneity, as they involve tumours from a single genetic background.

To mitigate for the harm associated with our model of choice, we will implement strict monitoring of animals that have undergone procedures. Additionally, the least harmful route possible for the administration of substances will always be used and analgesia will be used under guidance from the NVS. For our experiments, we require a sufficient tumour burden to represent the human condition and allow us to faithfully test novel candidate therapies. Therefore, implementing earlier endpoints than those described is not possible. However, we have included two protocols to minimise the harm to the lowest number of animals possible. One is focussed on the systemic tumour model and aims to map the disease progression in smaller animal numbers. This allows us to identify the point at which enough disease burden is reached to allow us to be treating with the

candidate therapies, while ensuring animal suffering is kept to a minimum. The second, is a tolerability testing protocol on a small number of animals to identify the maximum tolerated dose of novel compounds. This aims to ensure that in the larger efficacy testing protocols, the novel compounds are dosed at safe levels with the expectation that we would only observe mild adverse effects, and that any compound which has undesirable adverse systemic effects, at doses too low to be therapeutically useful, is abandoned before use in these larger cohorts.

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

Experimental models of human disease are essential for developing new treatments. The main challenge lies in using models that accurately mimic human disease, ensuring that effective therapies discovered in these models can be successfully translated into clinical treatments. Mice were selected because they provide a closer comparison to humans than less sentient model systems. Importantly, mice allow us to implant a human-like immune system, offering the necessary immune microenvironmental context for our candidate therapies. More immature life stages can not be used as mice must be maintained until they are adult in order to develop the humanised immune system that will be introduced.

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

Mice which have undergone procedures which are likely to result in adverse effects will undergo increased monitoring. Novel candidate therapeutics will undergo tolerability testing in smaller numbers of animals to determine the maximum tolerated dose with the endpoint being moderate clinical signs. Consequently, when we administer these drugs/molecules to larger groups of mice we do not anticipate greater than mild adverse effects. For tumour cells administered systemically, we will first conduct smaller scale studies to map the disease progression. This will ensure that we develop methodologies to accurately monitor disease burden so that animals do not suffer more than what is necessary to achieve our objectives. Importantly, these small-scale studies will grant us the opportunity to refine earlier endpoints where appropriate.

When using anaesthetics, type and depth of anaesthesia will be selected in consultation with the NVS. Furthermore, animals will be habituated (to handling and restraint procedures for example) wherever possible to reduce stress during procedures.

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

Turner et al 2011 'Administration of Substances to Laboratory Animals: Routes of Administration and Factors to Consider' J Am Assoc for Laboratory Animal Sci 50;600-613.

Workman et al 2010 'Guidance for the welfare and use of animals in cancer research' Br J Cancer 102;1555-157.

Ullman-Cullere et al 1999 'A Rapid and Accurate Method for Assessing Health Status in Mice' Lab Animal Science; 49(3):319-323.

As much as is possible, we will design and carry out our experiments in accordance with the PREPARE and the ARRIVE guidelines.

We will also refer to resources, such as webinars and publications from organisations such as LASA and NC3Rs, including the LASA report on avoiding animal mortality, LASA guide to aseptic techniques and NC3Rs newsletters.

The design of this proposal aligns with FRAME's guide as we have identified and implemented alternative approaches and where animal use was unavoidable we used their guidance for experimental planning including experiments designed to refine the design of larger experiments.

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

Regular updates from The Laboratory Animal Science Association (LASA) and the National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs) are distributed within our institution. These organisations supply educational materials focused on improving animal welfare and minimising harm and we encourage lab members to study these and to participate in NC3Rs webinars. Additionally, I hold routine meetings with my team to evaluate how we can adopt these best practices without affecting our experimental results.

We will also ensure that we follow the PREPARE guidelines to make sure our experiments are conducted in the most refined way possible.

66. The role of basal cell extrusion in invasion of transformed cells

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

cancer, invasion, metastasis, epithelial, extrusion

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?

We have discovered a new mechanism by which pre-tumour cells can invade early and independently of any primary tumour, suggesting a new model for how cancer spreads. We will test if this occurs in mice, using lung slices where cancer-causing mutations have been activated. Using different methods of microscopy, we aim to track invading cells live and seek a new way to prevent metastatic cancer, instead of trying to treat it.

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?

Metastasis is the reason that most patients succumb to cancer. Better understanding the mechanisms that initiate metastasis and the identity of cells and how they evade treatments would greatly improve our ability to diagnose and treat metastatic disease. The current thinking about metastasis is that it occurs after a primary tumour has grown and developed further mutations that allow it to break out of its primary site, suggesting that early diagnosis and treatment of a primary tumour would cure cancer. Yet, we know that some cancers are intrinsically metastatic and our ability to treat these is poor. Our work in zebrafish suggests a new paradigm for metastatic cancer where cells can invade early and independently of obvious tumours. If our modelling in mice proves to be correct, it could suggest a new approach of early blood screening and mild treatments that could target these escaped cells before they become cancerous to prevent metastasis rather than trying to treat it.

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

This work should contribute to several publications investigating the potential of our process to initiate metastasis in mice and likely humans. If this model holds true in aged mice, it could give us a platform for developing ways to screen for and treat escaped pre-cancerous cells before they become metastases.

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

In the short term, development of ways to film invasion events live within tissue slices could impact many researchers in cancer and other fields to help visualize disease progression with minimal harm to animals and to use fewer animals.

In the long term, our aim is to identify new ways to target invasive, but not yet cancerous cells to prevent metastases. Currently, doctors scan for primary tumours and then see if they have metastasized. Yet, once metastasized, these cancers are nearly impossible to treat. If we are correct, our approach would prevent metastatic cancer by implementing early screening of diffuse precancerous cells that would be easier and less toxic to treat.

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

We will maximise the outputs of this work through peer reviewed publication in open access journals, seminars and presentations at international meetings and conferences. Where appropriate, we will work with the communications teams at our Institute to disseminate our results more broadly to the public, for example, through press releases and lay talks. We will also maintain close links to industry partners and other external collaborators to ensure the most rapid translation of our work to patient benefit.

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.

Our first study visualizing cell invasion using this new mechanism we undertook using zebrafish embryos, in early development stages before they become protected by law. This approach was beneficial in allowing us to directly follow invasion events throughout the entire body and revealed that tumour formation and invasion of transformed cells could occur independently. However, for these studies, we used embryonic zebrafish, which likely behave very differently to adult tissue, which normally gets cancers. Additionally, zebrafish carcinogenesis may not reflect carcinogenesis in mammals for a variety of other reasons, as their genomes and environments are markedly different and they are not typically used for cancer models. Thus, in our next studies, we will test if 1) mammalian tissues do the same thing in adult (>3 months) mice, and 2) whether older tissue

(approximately >18 months, equivalent to onset of cancers in humans) responds the same way, since most cancers occur in older individuals. Thus, we have chosen to use well-established cancer models in mice to induce oncogenic transformations and film the ensuing events.

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

Because we are investigating only the early steps of transformed cell invasion, we will typically introduce lab-safe strains of viruses through the trachea to induce the expression of cancer-causing genes and, a few days later, harvest, slice, and image lungs from humanely killed mice. We will also allow some mice to go longer (up to 3 months) to see if we can detect disseminated cells before tumour formation by repeated non-invasive imaging with general anaesthesia to test whether putative therapies for targeting these disseminated cells, such as food withdrawal and chloroquine, prevent metastases.

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

Most of our experiments should have few adverse impacts on our mice, since we will principally be doing experiments on post-mortem lung tissue a few days following expression of cancer-causing genes and before the appearance of clinical symptoms of lung tumour or visible macroscopic lung tumour. Viral administration typically hits a small fraction of lung cells so only these will become affected by the activation of cancer-causing genes.

Based on results from our post-mortem tissue and cell culture experiments, we will eventually try to eliminate disseminated cells by allowing tumour development in a subset of animals. These potential treatments are commonly used and are meant to be very mild, so not expected to have serious adverse effects. However, two factors that may cause the most distress are fasting, which we plan to do for a minimum time and under strict supervision, as described below, and on aged mice, which will be monitored carefully.

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 our mice to experience only mild to moderate severity. In 90% of experiments, cancercausing mutations will be activated only upon administration of virus, and then will occur for just a few days before humane killing and tissue harvesting, which would be deemed mild severity. Genetically altered mice will similarly be of mild severity. Neither should impact breeding of the animals. Keeping older animals that should develop tumours without treatment will be deemed as moderate and these experiments will be done sparingly, in approximately up to only 10% of mice listed on our 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?

We have already demonstrated that basal cell extrusion can cause invasion of transformed cells in cells in culture, organoids, and in zebrafish embryos, but we need to test this now in an adult mammalian model animal. As mice are well-developed for cancer models, with many genetically modified strains already generated, they will be the most straightforward way to testing our mechanism in an older mammalian model in the context of the whole body, integrating the interaction between different cell types, as the complexity of these interactions cannot be faithfully reproduced in tissue culture systems.

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

We have and will continue to keep using cell culture models and organoids in parallel so that we are testing our findings from these systems in lung tissue slices. For instance, we will develop drug screens in tissue culture before confirming in mouse slices.

We have also investigated alternatives to using mice, such as through the 3Rs info hub:

https://www.3rsinfohub.de/organs/organ_lung/index.htmlhttps://www.3rsinfohub.de/organs/organ_lung/i ndex.html

Why were they not suitable?

The problem with setting up lung epithelial cells or lungs-on-a-chip is that they are unlikely to accurately model the real tissue, which we have shown greatly impacts the outcome of invading cells. We found that epithelial cell extrusion can be affected by both the mechanics and age of the tissue. Further, our previous work shows that cells that invade by aberrant basal cell extrusion can become different cell types that vary in their survival and response to chemotherapies, depending on the stiffness of the environment that cells later migrate through. Unfortunately, cell culture and organoids do not mimic real tissue environments well. Finally, many think that cells need to enter the bloodstream to

metastasize, yet our data in zebrafish suggests that cells can migrate in tight confines of the fascia, which was completely unexpected. Therefore, we need to follow these early events in their natural environments, as these factors will greatly impact metastasis and survival outcomes.

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 pilot studies with other labs using post-mortem ex vivo lung slices, we estimate that we will need ~1200 experimental mice (1020 adult and 180 aged) over 5 years to gather statistically meaningful data. The remaining mice are for breeding or do not have the desired genotype.

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

Our ability to use lung slices post-mortem enables us to greatly reduce the total number of mice used. Where other labs have conducted invasive live imaging of mice, as we are only investigating the earliest steps of invasion, we will limit our imaging to soon after expression of the oncogene in postmortem mice using lung slices. For each mouse, we can image and treat 12 separate lung slices from the same lung. This not only increases the robustness, as all drug treatments and control derive from the same lung, but it greatly reduces the number of mice.

We will develop our drug screening in cells in culture and organoids before treating ex vivo lung slices and, finally, in live mice. Thus, this will also reduce the number of mice needed.

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 do pilot studies in our organoid models before trialling any therapies to target invading cells. We may also harvest other tissues simultaneously to the lungs to test if this model is conserved in other tissues.

We will also constantly monitor animal use and archive lines by cryopreservation when not required over a period of time.

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 method of imaging uses only post-mortem tissue to ensure that the animals will experience minimal distress or harm. To obtain the tissue we will use adult mice to initiate lung tumours, typically by administration of viruses into the trachea to modify genes to induce tumour growth (oncogene expression). For some experiments we will need to use aged mice (up to 24 months of age), which may start showing signs of age, e.g. changes in coat colour, less active, for which we will adopt an additional monitoring regime to limit any adverse effects of aging. Tumour growth is unlikely to have many downstream adverse effects, as oncogenes will typically only be expressed up to a maximum of two weeks before humanely killing the mice. Eventually, we may implement fasting +/- other welltolerated therapies to test if they interfere with cancer dissemination after 3 months of oncogenic expression. In these cases, we will use the shortest interval of fasting for mice needed, based on our lung slice studies.

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

We have already done our preliminary studies on zebrafish embryos that are apparently less sentient. However, to confirm if these findings are relevant to human carcinomas that typically get tumours with age, we need to move to established mouse models for cancer. Using other model organisms like fruit flies or roundworms that are presumably less sentient would not be relevant to the objectives of this study for these reasons. As lung slices are not sentient, it greatly reduces harm.

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

Our experiments are already minimally invasive. We have also adopted a less hazardous way of inducing oncogene expression . Additionally, we will age mice only to 24 months and monitor for signs of aging starting at 15 months. We will typically do our experiments within a few days up to 2 weeks following oncogene induction to minimise harms.

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

We will adhere to the efficient breeding of genetically altered animals and will use the following publications to ensure that we are using the most refined methods:

PREPARE guidelines https://norecopa.no/media/7893/prepare_checklist_english.pdf

Procedures with Care https://researchanimaltraining.com/article-categories/procedures-with-care

Refining procedures for substance administration:
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3189662 and
https://journals.sagepub.com/doi/10.1258/0023677011911345

We will use the following to help refine our treatment of mice with respect to cancer models in rodents:

https://www.nature.com/articles/s41596-02400998w

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

Interactions with our nearby collaborators and going to conferences, reading the latest publications, and working with in-house biostatisticians and NIOs will help us improve our experimental designs to reduce the numbers required for breeding, and will help us to share tissue with other labs.

67. Central mechanisms of appetite regulation and neuroprotection

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

neurodegeneration, obesity, metabolism, neuroprotection, therapy

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 our work is to understand how appetite is regulated by the brain, and how genetic, chemical, and dietary factors acting in the brain can modify metabolic and neurodegenerative 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?

Obesity and the loss of brain cells (neurodegeneration) are common and often deadly diseases.

Obesity and its related diseases (like diabetes) affect over 1 billion people around the world, and is predicted to affect over 2 billon in the next decade according to the World Health Organisation. It leads to millions of deaths each year, and according to the Tony Blair Institute it costs the UK over 100 billion pounds each year in lost earnings and health care costs that take up 10% of the NHS's budget. Telling people to eat better and exercise has not proven to be an effective way for the whole population to achieve a healthier weight. While there are some new treatments for obesity like the drug

Ozempic/Wegovy that act on brain cells that regulate appetite, these treatments are expensive and have unpleasant side effects in some people like vomiting and diarrhoea, which limits the number of patients who could benefit. Surgeries to reduce body weight are relatively dangerous and cannot be applied to billions of people. Studies in mice therefore have the potential to identify new treatments that are more effective, have fewer unwanted side effects, and can address the global reach of this disease.

Neurodegenerative diseases like Alzheimer's Disease or Parkinson's disease can be fatal, and affect millions of people around the world and are a leading cause of death and disability among older people. There are often no effective treatments, so any treatment that could slow disease could have a huge impact. Work from our group and from others have shown that some treatments used for obesity or diabetes can also slow the loss of brain cells both in mice and in people. Our work will test which treatments are most effective in mice and therefore might also benefit people more than currently available treatments.

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

This project is likely to lead to new information, publications, and hopefully also new treatment strategies for common brain diseases. Specifically, we will study how brain cell types regulate appetite, and how factors that act on these cells can improve the health of mouse models of obesity. These studies are likely to lead to new datasets and publications. Factors that can treat obesity in mice could also be useful in humans, and lead to studies in humans. We will also study how candidate factors could slow the loss of brain cells in mouse models of neurodegeneration. These studies will identify which factors are effective, and how they might work, which could lead to the development of factors that are even more effective at slowing the rate of brain cell loss. Again, these studies could lead to ideas for treatments that could be used in humans. To maximise our chances of having benefit to people, we will also offer to work together with companies who are developing treatments, so that our expertise and models can contribute to new therapies.

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

In the short term, we aim to identify new brain pathways regulating appetite that may reveal new treatment strategies for obesity, and new treatments for brain diseases that lead to the loss of brain cells (neurodegeneration). This information is likely to benefit other scientists in a similar field of work, and also companies trying to generate new treatments for obesity and diabetes.

In the longer term, these insights can benefit the millions to billions of people worldwide suffering from obesity, diabetes and related diseases, as well as the millions of people suffering from the loss of brain cells each year. The diseases we are studying also cost over a hundred billion pounds to the UK each year, so our work could help save these costs.

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

We will openly publish the outputs of our work regardless of whether the results are the ones we expected to find or not, and make those findings accessible to everyone free of charge. Wherever possible, we will publish these results on ‘pre-print’ servers like BioRxiv to share them before they are published by a journal. To help other groups repeat our studies, we also openly share the methods we used to generate our results, and not just the results themselves.

We will also share our research results on social media like Twitter/X, at scientific conferences, and via public engagement events. Strategically, we work with a dedicated Public and Participant Involvement and engagement programme including dissemination of scientific advances with Social Media, engagement in Science Festivals etc. Our funders also have dissemination strategies to complement our internal channels.

More broadly, we are focused on allowing the "translation" of pre-clinical science into real advances in healthcare that can impact people's lives. There is also wide support for this with a specific office for translation keen to guide early translation pathways from pre-clinical models.

Species and numbers of animals expected to be used

- Mice: 6500 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.

To model human disease, it’s important to work with other mammals that have brains more similar to our own (rather than flies, for example). Mice are widely used to study brain disease, so our own studies can build on a vast amount of knowledge about their brains and behaviour. Since mice mature quickly, we can carry out our studies in a way that maximises the knowledge we will gain while minimising the number of animals we will need to study, and minimising the suffering of animals.

Specifically, since cell types involved in appetite and neurodegeneration are similar in mice and humans, results in mice are likely relevant to humans. Working with adult mice rather than newborn or juvenile mice allows us to ensure that any changes we see in their behaviour or body weight is not due to changes in their development, but is instead due to changes in the diseases we want to study. In some studies, we will use juvenile mice to induce the model of neurodegeneration, since injections into the brain can be done more easily and painlessly at this age than in adults.

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

Typically, we will use group-housed male and female adult mice to study obesity and neurodegenerative disease.

For studies of obesity, we will typically feed mice a high-fat diet for four weeks to make it obese, and then treated for two weeks with candidate factors (e.g. drugs) to test if they can promote healthy weight loss by monitoring body weight. We will include control groups that we feed a normal diet, and control groups that we treat with a non-active substance (called ‘vehicle’) to allow us to interpret the results of our candidate factor.

For studies of neuroprotection (slower or reduced loss of brain cells), we will typically induce neurodegeneration when mice are young by injecting their brains with a substance (for example misfolded proteins that induce neurodegeneration), and then treat mice with candidate therapeutic factors (like drugs used to safely treat obesity in humans) using the methods described above. We will then observe mice for several months to identify which factors keep them healthy for longer. We typically monitor them for signs of neurodegeneration such as loss of body weight and motor coordination (e.g. how well they can walk), and end studies before mice become very sick.

Less typical experiences that mice may undergo are listed below:

In some cases, we will feed mice different diets to study how this affects their body weight and other features of their metabolism or brains. In some cases, we will inject chemicals to model different aspects of human disease associated with obesity such as diabetes (high blood sugar) or hypertension (high blood pressure).

Typically, mice will have access to food and water whenever they want, but in some cases we will restrict access to food (fasting) for a short period of time (e.g. overnight).

In some cases, we will first test whether candidate factors are toxic before carrying out larger studies.

Wherever possible, we will deliver candidate factors via the food or drinking water. In some cases, we will inject mice via a syringe into the stomach through the mouth (oral gavage), under the skin (subcutaneous or SC),into the body cavity (intraperitoneal, or IP), into the muscle (intramuscular or IM), into the bloodstream (intravenous or IV), into the brain (intracerebroventricular, ICV). In some cases, we will implant mice with a pellet or small pump under the skin (SC) to deliver a drug at a constant level for a prolonged period of time, avoiding the need for multiple injections.

In some cases, we will surgically implant a small device under the skin to allow us to more easily identify mice (RFID tag) or track their movements (telemetry tag).

In some cases, we will also collect blood from mice to measure factors in their bloodstream, or test how mice respond to sugar (glucose) or the hormone insulin that causes sugar to be taken up from the blood into cells. These studies are called a glucose tolerance test (GTT) and insulin tolerance test (ITT), respectively.

In some cases, we will house mice in special cages that allow us to more easily measure how much food they eat, how much they move, and how much oxygen they consume.

In some cases, we will put mice into a special arena and take videos of their movement so that we can measure the way the move and the way they interact with their environment.

In some cases, we will surgically inject mice (typically ICV) with a virus that does not make them sick but allows certain brain cells to be labelled or genetically modified to test how these brain cells are connected to other cells, and how changes in these cells affect the behaviour or disease state in the mouse.

In some cases, neurodegeneration will be caused by breeding mice that have been genetically modified (e.g. transgenic), in which case we would not induce neurodegeneration by injection.

In some cases, we will image mice in special devices to study how their bodies are composed of fat, muscle, and other organs (DEXA scan), or to study the activity of their

brains or other organs (PET or fMRI imaging). This imaging is routinely performed in people and is non-invasive meaning that it does not require surgery or cause anything other than minor discomfort. Some mice will be injected as young animals with a substance that will cause neurodegeneration over the course of several months.

In the worst case scenario for studies of obesity and metabolic disease, a young adult mouse will be implanted SC with a telemetry tag, fed a high fat and high sugar diet, and be injected twice (several weeks apart) with the drug streptozotocin to induce a model of diabetes. It would then be moved to a metabolic cage for 72 hours to measure food intake and energy expenditure, followed by a DEXA scan, GTT, and ITT. It would then be injected every other day for 60 days with a candidate drug followed again by metabolic cages, DEXA scan, GTT and ITT before being killed.

In the worst case scenario for studying appetite-regulatory brain cell types, transgenic mice would be implanted SC with and RFID tag, be surgically injected with a candidate virus, and then fed a high-fat diet and monitored for body weight, food intake, and energy expenditure as described above before being killed.

In the worst case scenario for studies of neurodegeneration, a mouse will be injected with scrapie as a juvenile, then implanted with an RFID tag, injected IP every other day with a candidate factor for 60 days, monitored behaviourally in specialised arenas for behaviours, and allowed to progress until clinical signs of neurodegeneration have been reached before being killed.

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

Mice undergoing breeding protocols without undergoing procedures will be classed as reaching a "subthreshold severity" meaning no expected impact or harm. Most animals undergoing experiments will experience effects that are classed as mild or moderate severity. For example, a mild severity might be a mouse being fed a high-calorie diet to become obese, having a blood test or injection, and undergoing measures of memory and behaviour. Moderate severity will typically involve animals undergoing anaesthesia and surgery on brain. We expect their normal functioning to be disturbed after surgery for 2 or 3 days. They may lose weight during this time if eating and drinking less and may groom themselves less.

Some mice put on modified diets or injected substances to model obesity and metabolic disease will experience weight gain, high blood pressure, or elevated sugar concentrations in the blood for several weeks to months. Injections will only cause temporary discomfort. These conditions are also seen in millions of people and do not necessarily lead to suffering. Temporary fasting will increase hunger and/or thirst, but this procedure is routinely performed in people as well without causing adverse effects.

If candidate factors have never been used in mice before, we will first carry out ‘pilot’ experiments to ensure they are safe, staring with low doses in a small number of mice and then increasing doses and terminating experiments as soon as mice show signs of adverse effects.

Delivering candidate factors like drugs can cause temporary discomfort similar to that humans experience routinely. For example, vaccinations are given via intramuscular injections to people, and we will aim to use the least invasive method, such as subcutaneous injections or ideally delivering drugs via the food or drinking water wherever possible to reduce stress from handling and discomfort from repeated injections.

We will inject the brains of some juvenile mice with a substance that causes neurodegeneration. These injections are performed under anaesthesia, but do not require surgery. Some adult mice will be injected in their brains with drugs or viruses. These procedures require surgery that is performed under anaesthesia. Some mice have minor surgery to implant a device under the skin that can release a medicine slowly, or allow them to be identified or tracked. In all of these cases, mice are expected to recover quickly and will be given painkillers and post-operative care just like people recovering in hospital.

Similarly, blood collection is routinely performed in people and is not expected to have lasting adverse effects. Mice may also be briefly anesthetised to enable them to be imaged, as is sometimes performed for people undergoing similar treatment.

We will routinely house mice in social groups, but in some cases it will be necessary to separate them, for example if they are fighting (to prevent injury) or to carry out experiments in specialised cages for short periods of time. To reduce the stress of social isolation, and we will allow mice to acclimatise to their new surroundings and minimise handling. We also will sometimes move them temporarily to new surroundings to study their behaviour, which we do not expect to cause any harm beyond modest stress lasting approximately 30 minutes.

Imaging mice may require temporary anaesthesia lasting about 30 minutes, after which we will monitor them to ensure they make a full recovery. We do not anticipate these studies to have a lasting impact.

For studies of neuroprotection, mice will show no obvious signs of disease for many months, but will then experience loss of motor coordination and weight loss for approximately 1 month, but we will stop experiments before animals become too sick.

Rarely, mice may die unexpectedly after undergoing surgery or due to complications of neurodegeneration. In our previous licence this affected only about 1% of the total number of 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)?

We will only use mice, and do not expect any mice to exceed moderate severity. Remaining proportions are estimated to be:

Sub-threshold (e.g. breeding): 10%

Mild (e.g. injection or diet): 20%

Moderate (e.g. neurodegeneration), 70%

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?

It is essential to use animals since they can show changes relevant to disease like putting on extra fat, eating more food, or becoming less coordinated over time. These changes help us to understand if the treatments we are testing for metabolic and neurodegenerative disease in mice are likely to be useful to humans. There are some areas though where it is possible to avoid animal work, as described below.

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

Cells. We extensively use human cellular models, that allow us to reduce the total number of animals we use by ensuring that only the most promising approaches are selected. Specifically, we can work in a culture dish to turn human stem cells into some of the brain cell types that regulate appetite and are related to obesity, as well as some of the brain cell types that are affected in neurodegeneration. We can expose these cells to drugs and other candidate factors to test how they work in a culture dish. We can also generate more complex three-dimensional combinations of cells known as "organoids" that attempt to reproduce the complexity of groups of cells arranged into an artificial organ in a test tube.

Tissues: We can study human tissues (e.g. frozen brains) relevant for obesity and neurodegeneration by examining them under a microscope or examining the genes they express in single cells.

Humans: It is also possible to study the effects of candidate factors in people in ‘experimental medicine’ studies involving a few people, or in larger studies called clinical trials. We can gain insight into how these factors work by measuring food intake, hunger, energy expenditure, and by using noninvasive brain imaging methods like functional magnetic resonance imaging using high-powered magnets, much as we can also do in animal studies.

Why were they not suitable?

Cells. Cellular models allow us to understand how individual cells respond to candidate factors, but not how tissues or organisms respond. Organoids have potential, but still lack many of the cell types and interactions that are present in animals. Together, these studies will allow us to test out ideas in a culture dish before we have to use animals, reducing the number of animals we need to use, and refining the design of the studies we do.

Tissues: Human tissues are dead and do not allow experiments to be performed on them, but studying them can help us come up with better experiments to do in human cell cultures and in animals.

Humans: Before we can carry out these studies in humans, we typically need to know if the candidate factors are effective in animals. We also do not have access to human brain tissues, to understand how an experiment has affected cells in the same way that we can achieve using 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?

To estimate animal numbers, we consulted with experts and considered previous studies by our group and others so that we could obtain the most meaningful results using the smallest number of animals. We considered both how large effects in our experiments were likely to be, and how consistent results would be between mice. We will carry out many studies in both male and female mice to identify effects that are different between males and females. Where effect sizes are unknown (e.g. novel factors), we will first carry out 'pilot' experiments where we treat a small number of mice to determine if a candidate factor has an effect and how large the effect is, in order to better plan larger future experiments.

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

We have applied an online tool (the NC3R's Experimental Design Assistant) to help design studies and plan to continue using this too, and will consult with experts whenever needed. We also ensure that mice of the same age, genetic background (breed), and sex are used in the same experiments, and are housed under similar conditions. To ensure that the data we collect are as useful as possible, we collect it at the same time of day, and use electronic software to record the identify and the procedures we do on each mouse. Wherever possible, we will randomly assign mice to different treatment groups to ensure that groups are balanced, and only after performing an experiment and collecting the data will we reveal what group each mouse was in. Sometimes, separate people will assign mice to groups, collect data, and analyse data to ensure that the results we get are not accidentally influenced by our initial ideas. We also describe experimental methods in detail to allow others to repeat our work and build on it to make new discoveries.

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

We follow Home Office guidance on breeding GA animals to maintain efficiency and help optimise numbers used and the NC3Rs breeding and colony management resource (https://nc3rs.org.uk/3rsresources/breeding-and-colony-management).

Wherever possible, we will utilise refined and widely-accepted methods, such as feeding mice a highfat diet to generate models of obesity. We write up methods into standard operating procedures (SOPs) that we share with all members of the group to ensure that methods are carried out consistently and to a high standard to help ensure the success of our experiments using a minimal number of animals, and reducing the likelihood that studies will need to be repeated.

We will carry out most studies in a widely-used and well-characterised and genetically consistent breed of mouse (C57Bl/6J) rather than using transgenic mice. This approach allows us to build on the knowledge of many other groups when designing experiments to minimise animal numbers. It also reducesanimal wastage from transgenic breeding, and facilitate the generation of sufficiently powered experimental cohorts matched by age and sex.

Wherever possible, we adopt approaches that will maximise the data collected from individual mice, such as non-invasive behavioural analysis (e.g. food intake) followed by physiological analysis (e.g.

DEXA scan and GTT/ITT) and terminal biochemistry and histology (e.g. brain clearing and light sheet microscopy).

Where appropriate, we will perform initial ‘pilot’ studies to check feasibility and review the likely effect size and chances of a successful outcome before committing larger numbers of animals to a full study. Typically a pilot up to 8 animals will allow us to assess the technical feasibility of performing the study and to estimate the likely effect size and variability in outcome measures (hence an estimate of numbers needed for scientific outcomes). Finally, we collect and share tissues, and also share surplus animals where appropriate through the University's initiative to limit 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.

Choice of species and strain: We will work with male and female mice, typically housed together in cages to provide social enrichment. We largely work with mice that are not genetically modified and wherever possible work with the C57Bl/6J strain, a breed of mice that all have similar genes (like a pure-bred pet animal) since this strain is routinely used in studies of neuroscience and metabolic disease. Since many other groups have already worked with this strain, we can build on existing knowledge to design better studies and reduce the number of mice we need to use. Since many studies do not require genetically modified animals, we can minimise wastage of animals due to breeding.

Metabolic disease modelling: We will typically induce obesity by feeding mice a high fat diet since this approach is not harmful and is widely used, allowing us to directly compare our results to other studies in the field. This approach is preferable to genetic models of obesity (e.g. ob/ob mice) that can have features (e.g. diabetes) that reduce animal quality of life.

Neurodegenerative disease modelling: We typically inject the brains of young mice with a substance to induce neurodegeneration. After the injection, mice gradually start to lose brain cells within a predictable time frame. For the first approximately three months they show no signs of disease or discomfort, then after approximately one month of modest weight loss and loss of motor coordination (such as walking on uneven surfaces), and approximately one month later they show greater changes in their behaviours such as their memory and motor coordination . We have developed refined methods to measure these behaviours, allowing us to determine how far neurodegeneration has progressed quickly and accurately. We can therefore end experiments before mice experience any unnecessary suffering.

Experimental agents: When treating mouse models of metabolic and neurodegenerative disease with experimental agents, we will preferentially select agents known to be safe in humans. Wherever practical, we will deliver these agents in a manner that minimises pain and distress, such as via food or drinking water, or via a time-release pellet rather than by daily injections with a syringe. This approach both improves the experience of the animal, and generates more reliable data since the bioavailability of the experimental agent is more constant.

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

The aim of our research is to understand human neurodegenerative and metabolic disease (e.g.

obesity), so we require a model system that has cell types and behaviours similar to humans that are relevant to these diseases. Other animals that are commonly used in research, such as flies, worms, frogs/toads, and fish have different brain cell types and different behaviours, and would therefore be less informative about human disease. We also cannot use very young mice, since the diseases we wish to study typically affect adult humans.

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

We have made extensive refinements over the past years to minimise harms. For example, when we inject mice with substances like streptozoticin that can induce a diabetes-like state in mice, we typically use low doses that provide a more accurate model of that most common form of diabetes in humans, and reduce harm to animals. If mice become more sick toward the end of our studies, we supplement their normal diet with tasty and calorie-dense food and gel packs to allow them to eat and drink more easily.

Furthermore, we have established ways to monitor the behaviour of mice by video in our studies of neurodegeneration, allowing us to collect data at earlier time points, improve well-being of mice during the study, and terminate experiments earlier to avoid suffering. For example we gently take mice out of their home cage and put them into an arena that they can freely explore. After they have gotten used to the arena, we take movies of them to how much they move, how good their motor coordination is on a tricky surface, and how well they remember where an object in the arena was to test their memory. These studies are less stressful than regular observation and handling, the data we collect are not influenced by the opinion of a particular individual and are therefore more consistent, allowing us to use fewer mice and to end studies sooner. We use AI-based analysis of the movies to learn as much as possible from each animal, and as technology improves we can return to data gathered in earlier experiments to learn even more. To further refine these studies in the future, we will explore the use of continuous video monitoring of mice in home cages to eliminate any stress associated with handling or new environments, and providing richer data. Our aim is to carry out these studies in group-housed mice in home cages to allow for social and environmental enrichment and more natural behaviour. To achieve this goal, we would use ear tags or a similar method to clearly distinguish between individual mice.

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

We will use PREPARE (doi:10.1177/0023677217724823) guidelines when designing experiments and

ARRIVE guidelines (doi:10.1371/journal.pbio.3000411) when preparing work for publication. We will regularly check for updates to the Essential 10 and Recommended Set of guidelines

(https://arriveguidelines.org/arrive-guidelines). For survival surgeries, we will apply the LASA aseptic technique guidance “Guiding principles for preparing and undertaking aseptic surgery” (https://www.lasa.co.uk/wp-content/uploads/2018/05/Aseptic-Surgery.pdf).

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

To stay informed about 3Rs advances, we are subscribed to the monthly NC3Rs newsletter and will regularly review the NC3Rs resource library (https://nc3rs.org.uk/3rs-resources) and practical guidance from the Laboratory Animal Science Association (LASA). Furthermore, we will attend workshops, events, and webinars where these are relevant to our project plans, including those organised by our University. We will also look for advice from Named Person(s) about advances in 3Rs, and utilise our university’s tissue sharing resource.

To effectively implement advances, we update the SOPs used by the group to ensure that refined methods are incorporated into existing protocols. Where more substantial changes are required, we have a track record of regularly revising our current animal license to ensure that we can take approaches that reduce animal numbers and improve animal welfare, and we anticipate continuing this practice with the new licence.

68. Developing antimalarial intervention 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

Malaria, Mosquito, Vaccine, Drugs, Transmission-blocking

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 overall aim of this project is to better understand the basic biology of the malaria parasite, allowing furthering of antimalarial interventions such as vaccination and prevention of spread between the mosquito and mammalian host (transmission-blocking).

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?

Malaria is a life-threatening disease caused by some Plasmodium parasite species. The parasite is spread by infectious mosquito bite. The latest World Malaria Report (2023) reports 249 million cases of malaria in 2022, increasing from 244 million in 2021. Deaths remained steady at 608,000 in 2022 compared to 610,000 in 2021. Incidence and mortality remain high despite the wide-spread use of preventative strategies and antimalarial or transmission blocking drugs. With increased use of antimalarials, drug resistance is a significant problem limiting efficacy.

A first (from GSK) and second generation (from Oxford) vaccine have recently received licensure and been recommended for use by the WHO, however efficacy of the vaccines

remain low. There is a clear need for novel antimalarial interventions, including both novel drugs and vaccination strategies.

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

This project aims to generate the following outputs:

- The development of novel antimalarial and transmission-blocking drugs
- The development of novel vaccination candidates
- New insights into the malaria infection and transmission biology
- Publications and presentations (dissemination of data)
- At least one patent filed

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

In the short term, investigation of parasite genes of interest will lead to a more comprehensive understanding of parasite biology. Through publication and dissemination of data, this information can be used by other scientists with questions relating to these or related genes.

In the longer term, understanding these genes of interest can be used to generate novel antimalarial and transmission-blocking drugs that will benefit those that develop malaria or are at risk of becoming infected with malaria. Both the testing/development of antimalarial and transmission-blocking drugs and vaccination strategies will lead to long term benefits to those at risk of malaria, aiming to reduce and prevent malaria infection and subsequent transmission.

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

Outputs will be maximised by publication in open-access journals where possible and presentation of research at scientific meetings. Further, we will endeavour to pro-actively bring the attention of key stakeholders to our published findings, where they would stand to benefit from the research.

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.

This project uses adult mice, infected with Plasmodium parasites, a widely accepted model of human malaria infection. Parasites from a species of Plasmodium that infect mice are genetically similar to human malaria parasites and can be additionally genetically modified to express proteins identical to those in human infection, allowing the study of the disease in mice rather than humans. Like in humans, in the mouse host, the parasites initially establish asymptomatic liver infection, before proceeding to blood-stage malaria (symptomatic), and production of parasite forms that can infect mosquitoes. Without the

infection cycle proceeding through both the mosquito and mammalian hosts, completing the complex parasite life-cycle, parasites lose viability. Such an established disease model allows the study and development of anti-malarial drugs, transmission-blocking drugs and vaccination strategies. The use of mice is also required for the development of novel genetically modified strains of Plasmodium parasites that can be used to study the mechanisms of infection.

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

Generally, for parasite infection of mice, mice will be pre-treated with a drug called phenylhydrazine by injection. This drug promotes the production of red blood cells. Increasing red blood cell production increases the number of slightly immature blood cells that the parasite prefers to infect. This allows more rapid replication of parasites over a short period, reducing risk of developing unwanted sideeffects. The mice will then be infected with Plasmodium parasites, typically by injection of infected blood, or by infectious mosquito bite. The level of blood-stage infection (parasitaemia) is then measured by taking a small blood sample daily and assessing the percentage of red blood cells that contain parasites.

Where the aim is to produce infected mosquitoes, mosquitoes are then fed on infected mice under non-recovery anaesthesia.

Where the aim is to produce genetically-modified (transgenic) parasites, antimalarial drugs are administered to the mice to kill non-transgenic parasites, which do not carry resistance to these drugs, thus “selecting” for transgenic parasites. Transgenic parasites are then collected by taking blood of the infected mouse under non-recovery anaesthesia.

Where the aim is to test antimalarial and transmission-blocking drugs, drugs may be administered to the mice at any stage of the protocol.

Where the aim is to test vaccinations, typically a prime-boost regimen of vaccination occurs prior to infection of mice with parasites.

Mice will typically be humanely killed by a schedule one method unless the aim is to acquire parasites.

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

Mice infected with Plasmodium parasites are expected to exhibit limited adverse effects due to control measures undertaken in the procedures. For instance, an appropriate mouse strain will be selected for each experiment, limiting any adverse effects from blood-stage parasitaemia. This is because different mouse strains tolerate different levels of parasitaemia before developing symptoms. The protocols detailed in this project do not require the development of clinical signs, and so blood sampling allows a balance of reduction in animal numbers and refinement, culling animals before they become symptomatic.

Any antimalarial or transmission-blocking drugs used are not expected to induce adverse effects, and novel drugs will be initially introduced in a small pilot study after in vitro assessment and determination of cytotoxicity (damage to cells) and genotoxocity (damage to DNA).

Mice are expected to make a rapid and unremarkable recovery from general anaesthesia within two hours (as detailed under General Constraints). Adverse effects are also not expected from mosquito bites, as mice do not exhibit skin reactions.

Impact following vaccination prime or boost:

Mice may experience minimal pain from the vaccination procedures lasting in the realm of a few minutes and/or localised swelling at the site of vaccination that could last for several days. Depending on the vaccination platform, i.e., virus-based vaccines, piloerection (similar to 'goosebumps') may be expected, which would typically be less than 10 days, but may on occasion be prolonged (in excess of 10 days). Where piloerection is prolonged, mice will be humanely killed if they display additional clinical symptoms.

Vaccines are commonly combined with additives that enhance their effect (know as adjuvants). When use here they would be expected to cause brief (under 24 hours) local inflammation only due to use of the most refined options. In the case of some more potent adjuvants such as incomplete Freund's (the highly potent complete Freund's will not be used), a granulomatous lump (a small area of inflammation) may occur at the site of immunisation. On such occasions animals will be monitored following immunisation and any animals showing signs of distress such as prolonged abnormal behaviour or ulceration that breaks the skin 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)?

The expected maximum severity for each protocol is moderate. Across all projects, no more than 40% of all mice are expected to experience moderate severity, with the remaining 60% experiencing 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?

Due to ethical, logistical and safety restrictions its extremely difficult to conduct research using the pathogen which causes human malaria. In particular the stage involving infection of humans by mosquitoes which cannot be done without a living organism. Therefore, animal models are the only approach to studying this stage.

Rodent malaria is a safe (non-human infectious), versatile, biologically relevant and reliable model to study infection with malaria. In addition, there is currently no alternative to animal models with regards to vaccinology and drug sensitivity studies in a physiologically and immunologically relevant context

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

Complete replacement of the mouse model is currently not possible. However, we are working on developing robust and effective in vitro liver models to enable studies of the basic biology of the liverinfective stage, and drug sensitivity in an attempt at reducing our use of animals.

Given the essential role of live animals in maintaining mosquito colonies for malaria research, our institution has proactively sought non-animal alternatives for mosquito maintenance. Recognizing the necessity of blood feeding for mosquito reproduction, we have meticulously explored potential substitutes, particularly human or animal blood products. To inform this pursuit, we have leveraged scientific publications, databases and websites specializing in alternative methods for mosquito maintenance. Specifically, we have conducted extensive reviews of scientific literature, encompassing research articles, reviews, and conference proceedings, to identify alternative approaches to mosquito feeding that can minimize or eliminate the reliance on live animals while ensuring the health and reproductive success of mosquito colonies. Through these efforts, we have fully transitioned away from live animal feeding for mosquito maintenance, aligning with our commitment to animal welfare and advancing alternative methods in malaria research and adherence to the principles of the 3Rs.

Why were they not suitable?

Liver models are currently not robust infectious models and display limited use at this time. Further, they do not incorporate elements of the immune system that are required for vaccine and drug studies.

Parasites must pass through a mammalian liver in order to maintain viability and prevent chromosomal fragmentation. Repeat culturing of parasites in blood only will result in loss of the parasite.

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 has been estimated based on our extensive years of experience working on animal studies, allowing us to calculate the minimum number of animals required to achieve meaningful results.

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 proposed to be used under this project licence will be reduced by using wellestablished, robust systems (previously established in our facility under a previous PPL). The robustness of these systems greatly reduces the number animals used, as nearly 100% of animals become successfully infected upon inoculation. Our collective extensive experience of working with these systems enables us to reduce the number of animals used by careful planning of experiments, including appropriate controls and replicas thus avoid unnecessary experimental repetition.

For statistical/experimental design, in the majority of occasions, direct experimental measurements are not made on animals during the course of the procedure; instead, animals are used to generate parasite material for subsequent analysis. In these cases, the minimum number of animals needed to produce this material is used based on the acceptable minimum standards for publication of the results.

If statistical analysis is needed, the numbers of samples/experiments/observations is determined via consultation from the hundreds of available published studies, power calculations and using the most appropriate statistics with the possible aid of the NC3Rs experimental design assistant. In all cases, all experiments are designed to minimise the number of animals used, e.g. data from initial experiments is examined, and only if justifiable the full experiment is carried out (e.g. duplicate and triplicate). All analysis will be performed to encompass variation between individual animals and the non-parametric nature of parasitic distribution in the mosquito. The authors have extensive experience in this field and use standard methods accepted within the field. ARRIVE guidelines will be followed at all times.

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

The laboratory has migrated to more refined methods to provide blood for mosquitoes that does not require mice. In our prior PPL , this step required 3300 mice over a period of 5 years. Removing these steps from our experimental process is therefore a large step in reducing 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 mouse models under this project license.

At low to moderate parasitaemia (typically less than 20% of red blood cells infected with parasites), infections are well tolerated, causing little discomfort, with animals typically displaying normal, active behaviour. Some mouse strains can tolerate infection levels above 20%. Where possible, mouse strains with a tolerance for higher infection levels will be used to reduce the risk of symptomatic infection.

The procedures outlined here are engineered to significantly reduce the risk of long-term infection, associated with anaemia or infection of the brain (two adverse events that may be caused by parasite infection).

Where the aim is to generate genetically-modified parasites, adverse events are also avoided by using well-documented drug-treatment regimens where drugs are used to kill non-transgenic parasites.

Where the aim is to test vaccinations, adjuvants will be selected to minimise adverse effects whilst still allowing for an effective immune response. Adjuvants including Freund’s

Adjuvant, that are associated with excessive tissue damage, will not be used. Animals will be closely monitored for signs of complications at injection sites. Any lesions that do occur are expected to be minor and where appropriate will be promptly treated under the guidance of the NVS. Animals with lesions that are ulcerated or infected, or that do not respond favourably to treatment of minor lesions, will be humanely killed.

Where the aim is to test antimalarial and transmission-blocking drugs, animals will be given doses based on data derived from in vitro studies, and initial doses used are not anticipated to cause health problems since the drugs are pre-tested for cytotoxicity and genotoxicity. However, initial doses will also be tested in small pilot studies with escalating doses. Known antimalarials will initially be tested at doses equivalent to those already routinely used for humans and are unlikely to result in toxicity effects.

All animals will be housed in groups where possible, with appropriate environmental enrichment and fed according to current institutional 'best practice'.

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

For Plasmodium infection of animals and generation of transgenic parasites, the Plasmodium parasite must go through a mammalian host to maintain viability. Repeated blood-stage replication without passing through the mammalian liver-stage of the parasite life-cycle leads to chromosomal fragmentation and loss of the parasite. Mice are the least sentient animal that is a viable choice for the production of parasites. Further the protocols have been well established and refined over many years of use.

For evaluation of vaccine strategies, less-sentient animals do not have an immune response that is as representative of the human immune response or as well characterised in the literature.

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

Mice will be group housed where possible to reduce stress and related harm.

Environmental enrichment available within the facility will be utilised to reduce stress to the animals.

Mice will be monitored regularly post procedure to look for signs of discomfort and distress associated with immunisation at the site of administration as well as behaviour changes.

Sampling a small amount of blood to monitor parasitaemia allows for the culling of the animal before clinical signs develop.

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

LASA Guiding principles on good practice for Animal Welfare and Ethical Review Bodies.

LASA good practice guidelines - Handling and Restraint (Rat, Mouse, Guinea Pig, Rabbit)

Publication: “Refining Procedures for the Administration of Substances”, Morton et al. (2001), PMID 11201285.

Use of the website from the NC3Rs (https://www.nc3rs.org.uk) and LASA (Laboratory Animal Science Association) will also be made.

ARRIVE guidelines (Animal Research: Reporting of in vivo experiments).

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

We endeavour to stay up to date with the latest literature regarding the 3Rs. We shall work with the institutional animal facility to ensure that we are aware of the latest developments and implementations. We shall also ensure that all PIL holders take up the latest training available.

69. Regulation of T cell homeostasis and function in health and disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

T cells, homeostasis, immunity, viral infection, immuno-oncology

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?

Our aim is to identify and characterise the mechanisms regulating homeostasis and maintenance of the T cell compartment both in health and in disease 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?

We do not fully understand how T cells are maintained in an optimally functional state throughout the life course. New T cells are produced continuously throughout life by mechanisms of new cell generation and by self renewal of existing cells. Immune function declines with age, and it is unclear what cellular mechanisms are defective in aging T cells or the intracellular signalling pathways required for optimal function.

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

We will understand the dynamics of constitutive T cells generation contributes to maintaining a full and active immune system, how memory cells are generated and maintained and how memories to new infections and sorted and stored along side existing memories.

Our studies of how inflammatory signalling regulate development and maintenance of T cells will allow us to better predict how anti-inflammatory therapies influence the function of T cells and what impact this has on host immunity as a whole.

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

Understanding how T cell-mediated immune responses are generated and maintained has never been more important. Training the immune system to respond to pathogens through vaccination remains a front-line therapy, in the face of the ever growing spectre of anti-microbial drug resistance. Targeted T cell-mediated immunotherapy has finally come of age and is one of the fastest developing areas of cancer treatment. Vaccines and reactivation of anti-tumoral immunity depend on the generation of T cell memory. Therefore, understanding how T cell memory is established and maintained is fundamental for development of such therapies.

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

We aim to publish all novel, robust research findings consequent from the project, whether results represent new findings and insights or simply support a null hypothesis. This will be achieved by publication but also storing manuscripts with pre-print servers such as BioRXiv to maiximse speed of dissemination of new data.

We also collaborate extensively with mathematical modellers who perform sophisticated modelling meta analysis of data to extract the maximum information possible.

Species and numbers of animals expected to be used

- Mice: 15500

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 our research is to better understand how the immune system is maintained in a healthy functioning state throughout life. Because of the complex physical arrangement of the immune system, whole body model systems are required to unravel functions under homeostatic conditions as well in disease scenarios. While it is possible to generate information regarding regulation of some aspects of the immune cell behaviour in cell culture it is not possible to mimic homeostatic or immune responses to infection in vitro. The mouse represents a relevant and tractable system to study the immune system. Mice are readily amenable to genetic manipulation, which is a key tool exploited in this project. The immune system of the mouse, next to that of human, is the best characterised immune system of any organism, and for which there are the greatest number of tools and

reagents available to study. The natural life time of the mouse, at around 1-2 years, makes possible life long studies of immune function.

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

Typically, we study the function of immune cells following either genetic or pharmaceutical intervention. Transplantation of immune cells is also a key method to be able to follow the behaviour of a defined cohort of cells in the context of a normal immune system. As such, mice will typically be subject to drug conditioning, to allow injection of cells to become settled and established in a new host. Mice may then be subject to blood sampling to allow longitudinal analyses or killed to allow more detailed analysis of immune cell function. A single procedure would therefore typically involve 3-6 separate steps and monitoring continued for as short as a few days or as long as several months.

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

For the majority of experiments, analysing immune function following an intervention of some kind, mouse health and behaviour is not expected to be altered as a consequence. Infection of mice with specific infectious agents such as influenza virus, may result in transient weight loss, from which most mice recover. The condition of mice will be carefully monitored and weight loss not permitted to progress beyond defined limits, instead killing the mouse before severity limits are breached. Establishment of tumours is not expected to impair normal function or health. Nevertheless, tumour size will be strictly monitored to ensure tumour size does not exceed defined limits and result in impairment to physical function of 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 70% of experiments, we expect only a mild severity. For mice either subject to live pathogen infection or tumour engraftment, we expect severities to reach moderate levels.

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?

Because of the complex physical and temporal arrangement of the immune system, whole body model systems are required to unravel functions under homeostatic conditions as well in inflammatory scenarios. While it is possible to generate information regarding

regulation of some aspects of the immune cell behaviour in cell culture it is not possible to mimic homeostatic or immune responses to infection in vitro.

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

We considered using in vitro cellular immunology assay systems and immortalised cell lines.

Why were they not suitable?

in vitro systems, while useful, do not fully replicate the complexity of immune interactions or disease pathogenesis in vivo and it is essential to use appropriate and robust animal models to dissect these processes. Immortalised cell lines can be useful tools for testing broad principles or signalling pathways, the way in which signalling pathways are configured varies considerable between different tissues and indeed, the same cells at different stages of differentiation. Therefore, studying primary cells in vivo is critical to understand the conext specific functions we propose to investigate here. Furthermore to develop therapeutic approaches with potential to alleviate human disease it is necessary to establish parameters influencing efficacy in an animal model.

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?

It is difficult to predict the full course of experiments that will be undertaken in the course of pursuing a project over a five year period, as this depends on level of funding obtained. Individual experimental design are subject to power calculations to estimate the number of mice required for a single trial and then the number of trials required to provide statistically robust evidence of any differences. On past experience, with two large projects running, our breeding colony is around 60 breeding pairs for between 20-30 different genetic lines. This generates around 1000 mice a year that will be used for all purposes.

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

We have taken advice from statistical collaborators to take an experimental approach that aims to maximise the detection of experimental effects in a given trial, and then to test for reproducibility of experimental findings between trials. For mathematical modelling experiments, we use just as many mice as are required to allow robust estimation of model parameters. These are typically kinetic experiments and concentrating mouse sampling over the periods of greatest dynamics is critical for this. Conversely, fewer mice need be sampled in less dynamic periods. Such uneven distribution of sampling results in most efficient use of mice. Model fitting can also identify areas of uncertainty that would benefit from improved sampling frequency. Such iteration between experiment and modelling is a central principle in avoiding oversampling and therefore redundant use of mice.

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

Where ever possible, we use both male and female mice.

Breeding strategies for GM mouse lines ensure that, where possible, control groups can be taken from littermates or that control and experimental GM lines are derived from common parentage to ensure background genetic composition is equivalent.

Use of multiparameter single cell analysis methods permits several experiments to be performed and data collected from individual mice - studying more than one immune cell type from the same individual for instance.

Mathematical modelling is an integral methodology of our work. This informs optimal experimental design but also substitutes for empirical experimentation. Models make predictions that can be validated by much smaller experiments rather than large empirical experiments that might arrive at similar conclusions.

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 order to achieve our goals we propose to use mouse models to study the development and function of the immune system for several reasons. Mouse transgenic and knockout techniques are wellestablished and their haemopoetic system has been intensively studied and bears extensive similarities to that of humans. There exists a vast array of reagents facilitating the study of the immune system in contrast to the situation in other documented species.

Our procedures are of mild/moderate severity and have clearly defined endpoints.

However, it is not possible to fully predict the nature or severity of any potential defect and for all types of mice there will be careful monitoring for possible adverse side effects. Careful monitoring of strain characteristics will be employed and the information collated on databases to monitor whether phenotypes exceed their usual characteristics. Animals exhibiting any unexpected harmful phenotypes will be killed using a Schedule 1 method, or in the case of individual animals of particular scientific interest, advice will be sought from the local Home Office Inspector.

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

To our knowledge no other species of lesser sentience can fulfil the requirements of this programme to the same extent as the laboratory mouse. The composition, structure and function of the mammalian mouse immune system closely resembles that of human, and

can host many of the same pathogens that cause human disease making them ideal for studying immune responses. Lower vertebrates adaptive immune systems exhibit substantial differences to human, while invertebrates lack adaptive immunity altogether.

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

We have gained a detailed understanding of the genetic and chimeric mouse systems employed in this project. We have specifically developed the use of the conditioning drug busulfan to allow generation of bone marrow chimeric mice. This represents a substantial refinement of the traditional conditioning methods of lethal irradiation of mice, which have numerous well documented welfare costs to mice. Drug treatment is non-lethal and, at the doses employed experimentally, very low toxicity.

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

When designing and planning new experiments, we apply the 15 point PREPARE guidelines (https://norecopa.no/prepare) to guide us through the processes of study formulation and quality control, and for discussion with our local biological services staff.

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

We remain alert to technological advances that permit refined use of mice or replacement with in vitro model systems, as well as monitoring the 3Rs literature and newsletters.

70. Blocking senescence in ageing

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

ageing, senescence, anti-ageing, senotherapies, senolytics

Animal types	Life stages
Mice	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?

To assess the importance of cellular senescence in ageing and to test the biological effects of preventing senescent cell accumulation on healthy ageing.

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 is important to help us understand how and why ageing develops and what is the importance of the accumulation of old (senescent) cells in age-related pathologies (such as

Alzheimer's, sarcopenia, frailty, heart failure or cancer). Also, we will study new therapeutic approaches to ameliorate ageing, based on the results obtained under the previous project licences, which identified several potential anti-senescent strategies.

We will use different mouse models to understand the mechanisms responsible for normal and pathological ageing and their associated diseases, and to try to explain why some people age better that others and have longer lifespan. Our experiments will facilitate the design of new tools to study ageing and, eventually, to promote healthy ageing in humans.

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

We expect to determine whether different approaches to reduce the accumulation of senescent cells (collectively known as "senotherapies") can be used in vivo to ameliorate age-related pathologies and enhance healthy ageing. Also, we want to determine whether new markers could be used as diagnostic/prognostic tools in ageing. Our in vitro experiments will produce information on new compounds and interventions that could prevent, reduce or reverse senescent cell accumulation in tissues and have an effect of mammalian lifespan/healthspan. This will lead to new publications and, potentially, provide the basis for clinical trials.

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

Studies of ageing in mouse models are indispensable and complementary to other research experiments. These models are important to understand how and why ageing develops in an organism and which is the impact of senescent cells in the development of age-related pathologies such as cancer. We would like to use existing mouse models that will recapitulate what is observed during normal human ageing, as well as in premature ageing syndromes such as progeria. This will help us to understand the mechanisms responsible for normal and pathological ageing and their associated diseases and may help us to explain why some people develop age better that others and have longer lifespan.

The short-term benefits of our research will impact the community of scientists studying senescence ageing and age-related diseases, including the generation of new tools for senescence research. Also, the researchers working on this project will benefit by being trained in state of the art techniques and protocols by leading experts. A mid-term benefit will be that companies in the commercial private sector could engage in testing the approaches suggested by our research. This could foster the economic competitiveness of national enterprises and attract R&D investments from global business. In the long term, our experiments will facilitate the design of new therapeutic/diagnostic/prognostic tools to improve healthy ageing and reduce the impact of age-related diseases in humans. Thus, they would benefit geriatric patients suffering common conditions such as cataracts, diabetes, Alzheimer's, cancer, frailty, etc., as well as those having premature ageing syndromes. Many of the diseases have no cure and preliminary data suggests that they could greatly benefit from anti-senescent strategies. The tools derived from the knowledge generated in this project could have an impact on globally enhancing quality of life in the future.

We have already generated data describing a new type of drugs with potential anti-ageing effects, which, in this mouse model, showed a protection against age-related cognitive decline. This pre-clinical data can inform future clinical trials to translate these findings in humans. Similar data with other drugs is now being analysed. Moreover, we have generated a nanotechnological tool to detect old cells in vivo, which could have diagnostic applications. These are examples of the groundbreaking results that can be obtained in this project and their potential impact on human health.

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

We have several collaborations with scientists working on the field of senescence and ageing, as well as biotechnology companies. We will use their expertise to take our results forward and test any compounds in other models, in order to accelerate any translational applications. We will also focus on the public dissemination of our results, with publication of articles in specialist journals, presenting results in conferences, networking with focused groups and engaging a wider audience through activities aimed at the public dissemination of science (such as open days and lecture series).

Species and numbers of animals expected to be used

- Mice: 1820

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 ageing in humans, it is essential to use a mammalian model. Lower animals (flies, worms...) are often used in ageing research, but they can only provide a partial view of the processes that are common in most mammals, including humans. Within mammals, mice are the most appropriatemodel, for being the most commonly used and well studied, efficient and practical. Mice will be studied over their whole lifespan to follow all the stages of the ageing phenotype.

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

Genetically modified mice, which display an accelerated ageing phenotype, will be studied.

Heterozygous animals, with no expected adverse effects, will be bred and the resulting homozygous animals will be further studied. These will given drugs (by oral gavage, tail vein injection or other methods, as appropriate) and changes in lifespan/healthspan will be recorded by measuring a frailty index over their whole lifespan or a period of time. Functional tests may be performed to assess amelioration of specific age-related symptoms (e.g. Barnes maze to test spatial memory, Kondziella test to measure muscle strength, functional imaging for heart function, etc.) and blood samples may also be obtained, according to the requirments of the differnent experiments.

Animals will be dosed starting after 2 months of life and kept in the protocol until the endpoints are achieved. A single animal will not be subjected to all the optional steps described above. Involvement of each animal in one or more optional steps will be

carefully determined to minimize stress. Animals are likely to be involved in 2-3 of these steps maximum for most of the experiments.

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

In most of the steps, no adverse effects are expected, except those associated with ageing. Many compounds and interventions to be used have already been tested in mice and shown to produce minimal side effects. Some agents may cause discomfort (e.g. local injection site reaction) when given repeatedly. The likelihood of such an event is extremely low. We are not anticipating the increased appearance of tumours in the mice used in these experiments. Mice may develop reduced mobility as they age, accompanied of a general reduction in organ function (which can lead to dental disease, corneal opacity, dermatitis, lameness, seizures and other age-related symptoms).

Although unlikely with the types of drugs used for the experiments described here, adverse effects should always be expected when using therapeutic approaches that have not been reported before. Thus, these new compounds will be tested in small scale pilot studies to determine toxicity and potential side effects before being used in the relevant steps.

In our experience, these mice can sometimes experience a chronic (over weeks) weight loss of >20% of their weight without displaying any signs of ill health, as this is part of their normal ageing phenotype. However, acute (between 2 health checks or over 3 days) weight loss of 15% or more would be taken as a sign of ill health and the mice will be immediately humanely killed by a Schedule 1 protocol.

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/moderate for all animals (likely to be 50% mild, 50% moderate).

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?

Cell cultures cannot recapitulate the response of a full organism when it comes to ageing. Some preliminary tests have also been crried out on flies but, although genetically similar to humans in many aspects, they are evolutively too distant for experiments done on them to be conclusive in terms of human applications. Non-mammalian models, such as fish or C. elegans, have limited interest for advanced studies due to the huge differences in the

ageing processes between these species and human. Thus, this is why experiments in a mammalian model such are mice are necessary in order to bring these compounds to potential clinical trials.

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

All preliminary experiments to test the biological effects of the compounds to be tested in mice are usually first done in mammalian cell cultures, and only the ones that show potential are taken forward. Organismal ageing can only be studied in animals, since cells or in silico models do not recapitulate the full phenotype. Initial experiments were also done first in flies, but the fact that mammalian ageing is substantially different from the one seen in insects forces us to use mice (or a similar animal) to obtain meaningful pre-clinical data.

Why were they not suitable?

The relevance of cell culture models to assess organismal ageing is very limited.

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 based this estimate on the mice used during the previous licences related to this project and the power calculations performed specifically for this project. In our previous work, we tested different drug concentrations of different anti-senescent drugs. In this project we will use the doses that showed least side effects, for further work with these drugs, thus reducing the number of animals needed for preliminary tests. We have used online tools (see below) to estimate the number of animals needed to achieve significance using ANOVA tests.

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

We used the advice of in-house experts and the following online tools:
https://www.statisticshowto.datasciencecentral.com/
http://www.quantitativeskills.com/sisa/calculations/samsize.htm

NC3Rs' 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?

Breeding will be carefully controlled to avoid surplus animals. Data from previous studies will be used to determine the best drug concentrations and dosage schedules. When using new anti-ageing drugs, pilot studies will be performed first to determine the best doses.

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 normal and fast ageing mice, as well as other models of age-related diseases. Working with fast ageing mice allows us to reduce the time the animals are kept in the study. We will assess the lifespan/healthspan changes in the animals using a frailty score, such as the one described elsewhere in this document. The animals will suffer no harm or distress, other than that associated with normal ageing (preliminary experiments show that the side effects of the compounds used are minimal) or potential unknown side effects when using new anti-senescence drugs (risk of suffering will be minimized by testing these drugs first in a small pilot study).

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

Although useful for preliminary studies (for instance, we first tested some of our drugs in flies), nonmammalian models are not adequate for pre-clinical studies of interventions in humans since they do not fully recapitulate human ageing phenotypes.

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

The animals will be health checked at least once a week as part of the frailty assessment (a test where we measure age-related parameters such as locomotion, hydration, muscle tone, etc.). Given the fact that distress signs are an essential part of the ageing phenotype, we will already be monitoring very carefully their appearance during the course of the study, due to the intrinsic experimental value of the data. Interventions will be immediate if any threshold is reached, thus minimizing the harm to the animals.

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

We will use the PREPARE and ARRIVE guidelines for planning and reporting experiments, as well as the LASA dosing and blood sampling guidance.

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

Throughout the duration of the project, we will always look at advancing the 3Rs with everything we learn from reading the literature, users meetings, and other forums. Particularly, we will review the

NC3Rs, FRAME, NORECOPA, RSCPA websites to keep us informed of advances in 3Rs relevant to our work. Moreover, we will be assisted by the staff of the animal facility, who

will inform us of any changes that could be made. Also, the NIO circulates a Newsletter that incorporates up to date information from the NC3Rs website, which will be helpful to keep us updated.

71. Mechanisms and drug action in atherothrombotic 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

cardiovascular, thrombosis, blood vessels, pharmacology, 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 of this project is to understand how processes in our bodies, medicines we take and pollution we are exposed to can damage our heart and blood vessels. We want to do this to find new medicines to prevent heart attacks and strokes, so we can improve the safety and effectiveness of existing medicines and protect the public from harmful pollutants in our 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?

Cardiovascular disease is the biggest killer in the UK and worldwide and happens when our hearts and blood vessels become damaged. We have lots of effective medicines and treatments that help slow the disease, but heart attacks and strokes remain all too common. To help prevent more of these we need to understand better how our hearts and blood vessels become damaged either through processes in our bodies or external factors. To do this, we are looking at three main areas:

(1) Pathways in our body that are switched in our blood vessels during cardiovascular disease. Our research looks at two of these pathways, a protein called RGL1 and a family of hormones called prostaglandins. We believe new medicines that block these pathways could help prevent and treat cardiovascular disease. Blocking them may also have benefits in other diseases like sepsis and cancer.

(2) Damage to our hearts and blood vessels as a side effect of medicines taken for other conditions. We are studying two types of medicines, ibuprofen-like drugs called NSAIDs which are used to treat pain and inflammation, and anti-retroviral drugs which are used to treat and prevent HIV. Whilst they are very effective, we know these drugs can also increase the risk of heart attacks and strokes. We want to find ways to prevent this or to find new safer alternatives.

(3) Pollutants in our environment that can damage our hearts and blood vessels. We are focussing on microplastics which are tiny plastic particles found in our air, food and water and which we think can build up in our blood vessels and damage them. We want to understand this better so we know which plastics are most dangerous and learn how we can prevent this through medicines or regulation.

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

The ultimate aim of this project is learn how processes in the body, side effects of medication and pollution we are all exposed to can damage our hearts and blood vessels to cause heart attacks and strokes, and how this can be prevented. We hope to find new ways to treat these diseases, new ways to use existing medicines more effectively and safely and new ways to protect us against pollution. To start, this will be in the form of new scientific knowledge which will be published at the earliest opportunity in scientific journals and presented at scientific meetings. For example, under our current project licence we have published 8 papers in some of the leading international scientific journals in our area of research and regularly presented findings at meetings of scientists and medical staff. We will always keep in mind how to use this to help patients as quickly as possible. By the end of the project we hope to be able to understand enough to begin to develop some of the following:

1. New medicines that block RGL1 or harmful prostaglandin pathways in our blood vessels to prevent heart attacks and strokes and protect or treat other types of disease like sepsis and cancer.

2. New versions of ibuprofen-like drugs (NSAIDs) that are safer, especially to the heart and blood vessels or medicines that can be given along side them to protect against heart attacks and strokes in people living with arthritis and pain.

3. New approaches to prescribing antiretroviral drugs to people living with HIV, avoiding types that can damage the heart and blood vessels and replacing them with safer versions.

4. Predict which types of microplastic pollution are the most damaging to our hearts and blood vessels so they can be removed from our environment by changes in plastic manufacturing or regulation.

The knowledge gained and publications generated in this project will only be the beginning but will be an important first step in each of these areas. Along the way we also hope to learn things we weren't expecting to find which may include both new scientific discoveries and new ways of performing experiments to refine, reduce and replace the use of animals for medical research experiments.

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

In the short term (0-5 years), we will generate new knowledge that will help drive scientific research. We will test our ideas and find new targets that can be the basis of medical tests and treatments to help patients. In the medium term (5-10 years), we can follow this work with studies on humans and patients with disease. In the longer term (10+ years), if these are successful we will be able to turn our basic scientific research into real benefit for people living with cardiovascular disease, arthritis and HIV. Almost 20 million people in the UK live with one of these conditions so any new medicines or ways to improve existing ones will have an important impact.

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

Maximising the outputs of any scientific research depends on communication and collaboration with other scientists and medical staff and keeping the public informed of any progress.

Scientific communication: We will communicate our findings to other scientists as soon as we can and in the highest profile ways possible so our colleagues will be able to contribute to the research. This will include publications in scientific journals and presentations at meetings of scientists who work in the area. Importantly, we will make sure all our findings, either positive or negative are shared so that even if we are wrong about some things, other people can learn from this.

Collaboration: We will closely collaborate with other scientists and medical staff to develop the work in the fastest most effective way possible. At the earliest opportunities we will begin to work with medical staff, with drug companies and with government bodies to develop our scientific results into new ways to help people living with health problems and protect the public.

Public engagement: We will also make all efforts to keep the public informed. We will send press releases about things we discover and directly interact with members of the public through events organised at our universities that are open for everyone.

Species and numbers of animals expected to be used

- Mice: 8000

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 mice for this project because they can be easily genetically manipulated and are lowest species for which refined models of cardiovascular disease and inflammation have been established.

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

Mice used in this project will either be wild-type or carry genetic modifications which are scientifically important but are not expected to impact the animals welfare. These will be used in the following typical protocols:

(1) Genetically modified animals will be humanely killed and samples of their organs and tissues taken for study without any other procedures being performed.

(2) Animals will receive changes to their normal food, be treated with medication or receive microplastics in their food/water, by injection or through a feeding tube. This could be a single time or on a regular basis over several weeks. This would generally not impact the animals welfare beyond a short discomfort associated with an injection. In rare cases, small blood samples may also be collected from the animals tail.

(3) Animals will receive an injection of a substance that can cause an inflammatory response which causes them to feel unwell (strong flu-like symptoms) for up to 2 days.

At the end of each protocol, animals will be killed humanely straight away or deeply anaesthetised for short surgical procedures to study their heart and blood vessels during which they will not suffer and from which they will not be allowed to wake up.

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

We have worked hard to design the best possible experiments that will have the minimum effects on animal welfare. Although each protocol in our project carries a severity limit of 'Moderate' this is a 'worst case scenario'. In the majority of cases animals would not be expected to experience more than minor and brief discomfort, for example, a short pain associated with an injection. Where this is exceeded it would be because although each step that animals experience might only have a small impact such as a short pain, many of these steps may be done in the same animals so together over time they may add up to a bigger effect. We also have one protocol where animals would be treated with substances that cause inflammation and we know this could cause them to experience strong symptoms of an infection where they feel generally unwell. We would not allow them to suffer in this way for more than 8 hours for strong symptoms or 2 days for milder symptoms.

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 (mice) 10%

Sub-threshold (mice) 60%

Mild (mice) 20%

Moderate (mice) 10%

Severe (mice) 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?

We need to use animals in our project because cardiovascular disease and inflammation are complex processes that involve many different tissues and cells working together. We don't yet understand all these processes, which cells and parts of the body are involved and how they cooperate so unless we study animals as a whole we cannot learn how to prevent and treat these conditions.

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

In our work we use many different types of experiments to answer our scientific questions. This includes experiments on cells grown in the laboratory, experiments on blood and tissue samples taken from healthy people and people with disease and some experiments that are performed virtually using computer simulation or computer analysis of online data. Wherever we can we will use these approaches to replace experiments on animals.

Why were they not suitable?

Unfortunately, we cannot always use simpler approaches in the laboratory with cells or tissues because until we understand the basic processes of diseases we don't know which cells and tissues to study and how to model them. Once we understand these things better we can and will move to replace whole animal experiments with other experiments that don't involve animals.

Another reason we can't always use simple non-animal alternatives in the laboratory is that they cannot show us how all the tissues in the body cooperate to control disease. As an example, we know that cardiovascular disease involves the heart, blood vessels, the kidney and cells in the blood. We can study these separately in the laboratory but unless we study whole animals we cannot see how they work together.

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 in this project by performing statistical calculations to work out how many are needed for each experiment and thinking carefully about the total number of experiments we will need to perform. These estimates of animal numbers have also been reviewed by other scientists who work in the area to give us confidence in them.

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

We carefully plan all our experiments to minimise the number of animals we need to use and get the most scientific information possible. This includes looking at experiments we have done in the past to see how these can be improved and animal numbers reduced, using tools like the NC3Rs
Experimental Design Assistant to plan and organise all parts of the experiment, and taking advice from biostatisticians and other experts in our institute.

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 take all reasonable steps to reduce the number of animals used in our project. Most of the animals used will be from genetically modified colonies which we will breed carefully at expert facilities in ways that minimise waste and ensure that every single animal can be used in experiments. We will start all our experiments with a small pilot of a few animals to be sure that there will be no unexpected welfare harms and to see if it is needed to go on to a large experiment with more animals. We will collect as much information as possible from every animal for example, making many measurements from the same animal over time. We will also collect tissues from all our animals, and share with other researchers, so no additional animals are 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.

We will use several different models to induce cardiovascular disease and inflammation and study how these effect the function of the heart and blood vessels. We will use simple models of coronary artery disease where mice are given added fat and cholesterol in their

diet. These are in the majority of cases mild models so don't cause distress or lasting harm and animals will be killed before they experience any adverse effects of the disease. In rare cases, multiple steps that have a small harm may be needed in individual animals which could add up to cause a moderate welfare harm overall. This would be only done where unavoidable. We will also use models of inflammation where mice are treated with substances that mimic a bacterial or viral infection, which can cause symptoms of an infection like flu. We use this because compared to other models it is very easy to control so animals experience the minimum suffering for the shortest time. At the end of protocols, we may use surgical procedures that let us directly and accurately measure how the heart and blood vessels function but this will only be done under deep anaesthesia from which animals will never wake up alongside strong pain killers so they don't experience any suffering or distress.

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

We will use mice in the simplest and most humane models possible. In most experiments, animals will be humanely killed without any harmful procedures or procedures will be carried out under deep anaesthesia from which animals will not wake up. We chose to use mice because are the least sentient species in which it is possible to manipulate how genes work, to model the diseases we want to study and measure the function of the heart and blood vessels in ways we need to.

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

We have carefully chosen the most refined models that can deliver as much scientific value as possible with minimum amount of suffering. We will make all efforts to make animals comfortable whether they are undergoing an experimental procedure or not, for example, giving them a rich environment to live in and handling them in ways that don't cause them stress. During any experiment, we will closely watch all animals for any sign of discomfort and distress and either stop the experiment or humanely kill any animals suffering unexpectedly. Wherever some suffering is expected from an experimental procedures we will make all efforts to reduce this for example, in the case of animals with inflammation, by keeping them warm to reduce their symptoms or in protocols where animals will be handled a lot, we will get them used to people beforehand to reduce their stress levels. Through the project we will continue to look for new ways to refine our experimental protocols and make animals more comfortable.

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

We will always aim to follow the best possible practice in performing any kind of experiment including those on animals. Although there is no specific guidance published covering the models we use, we will always search and review published information to find the best ways to perform our experiments. More generally, we will follow published best practice guidance for how to design, perform and report experiments to the highest standard including the NC3Rs PREPARE and ARRIVE guidelines and the Centre for Open Science Transparency, Openness and Reproducibility (TOP) guidelines.

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

We will constantly review our protocols and experimental design to reduce, refine and replace the use of animals. We will keep informed about any advances by reading scientific publications and speaking with colleagues within our own universities and elsewhere. This will include not only other scientists but those directly involved in the care and welfare of animals. In our university there are also frequent meetings and training events to discuss the latest developments in the 3Rs and how to implement them. We also subscribe to the NC3Rs newsletter to keep up to date with the latest developments in these areas externally.

72. Somatic mutations in organ regeneration, ageing and 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

Somatic mutations, Regeneration, Stem cells, Ageing, Cancer

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 project aims to discover the role of genetic mutations in biological processes involved in tissue injury, repair and regeneration and the links to 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?

With the advent of modern technology and medicines over the last 50 years, we are now able to live longer but as a result, the incidences of chronic diseases and cancers are increasing across the population and there are currently few effective treatments or cures. Recent scientific developments have demonstrated that genetic mutations accumulate in

all cells over time and some of these mutations have profound and unexpected effects on the behaviour of cells and organs. Often these genetic effects may be linked to chronic diseases in multiple organs such as the consequences of obesity contributing to diabetes, liver disease and cardiovascular disease. Understanding and harnessing these genetic and biochemical processes, for example through new drugs, will enable us to successfully treat a range of diseases thereby significantly improving the health of the population.

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

The project will generate significant amounts of new information on how genes function and how they interact in causing disease. There will also be outputs of large-scale datasets such as DNA and RNA sequencing data. All results will be published in peer-reviewed journals, with an emphasis on open access, and the data will be made widely available to ensure maximum benefit across the scientific community. The project may also generate new materials and transgenic lines.

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

The impact of this project will be substantial since the focus of study, that of the effects of genetic mutations on cellular physiology and pathology, may be widely applicable to many scientific disciplines. For example, discoveries on the genetic networks central to cell proliferation and organ regeneration, may be applied to understand and prevent cancer progression. The long term of this project is to translate these discoveries eventually into novel treatments or preventative strategies, in order to benefit patients and to reduce the health burden across the entire population.

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

A priority is to ensure that the results from this project are widely accessible and disseminated across the scientific community. This will take the form of collaborations, presentation at scientific meetings, uploading and sharing of pre-prints and an emphasis on publications in high impact, open-access peer-reviewed scientific journals, including that of negative results. Datasets will be archived long term in major data repositories and made available to researchers. New bioinformatic codes will be made available through collaborations and uploaded onto Mendeley. New materials or transgenic lines will be shared under appropriate material transfer agreements.

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.

Biological processes such as ageing and cancer are closely connected to mechanisms of organ repair and regeneration following damage. This project focuses on the contribution of genetic mutations to these mechanisms and how mutated cells interact with surrounding

cells, such as immune cells. The mouse is the animal model of choice in these type of studies due to the small size, short-breeding cycle and wide range of genetic and experimental tools available. Indeed much of the body of scientific knowledge accrued over many years has been based on mouse genetics which have shown that a constellation of genes are turned on (or off) at defined timepoints from the embryo all the way through development. Some mutations alter the activity of some of these embryonic genes in adulthood and this is thought to be a major contributor to cancer and the ageing process. Given the interconnectivity of these genes therefore, this project will use all life stages of mice from embryonic through pre- and post-natal development to aged mice.

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

The aims of the project are to explore how certain genetic changes affect the physiological response of different abdominal organs to damage. Typically a mouse will be engineered (for example through selective breeding) to carry genetic mutations either throughout all cells or just in an organ of interest. The subsequent effects on that organ through development will be explored, as well the repair mechanisms following organ damage. Broadly, a range of injuries will be studied which are common in the general population, such as high fat diets resulting in diabetes and fatty liver disease, and which therefore reflect real-life clinical scenarios that affect patients in order to understand more about the processes of organ repair, regeneration and ageing.

Some injuries will be over a short period of time (typically one week) such as the administration of toxins or short operations via an abdominal incision (typically one hour). Other injuries will be typically over a long period and may involve specific diets, for example those with high sugars and fats, which are processed by the gut and cause damage to organs such as the liver and pancreas over a period of time (e.g. 12 months). We will study how ageing affects the ability of organs to repair themselves following the same type of damage. In addition to studying the way these factors affect specific organs, there will be experiments to test different methods to either prevent the damage in the first place or better enable the body to heal following the organ damage. Such methods may include delivery of replacement cells generated in the laboratory, genetic material using viruses (i.e. a form of gene therapy), or new drugs that have been discovered. Repair of the damage to an organ will be assessed typically through techniques such as blood tests and imaging modalities such as non-invasive CT scans.

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

Part of this project will be observational to assess the changes to abdominal organs such as the gut, pancreas or liver when genes of interest have been mutated. There will be minimal adverse effects in the majority of the animals, however some may develop tumours which could manifest in signs such as weight loss. In the part of the project which focuses on organ repair and regeneration, the animals will be challenged with toxins, dietary changes or surgery (e.g. resection of part of an organ) and experience adverse effects, mostly lasting only a few days, such as abnormal behaviours or reduced activity, weight loss and temporary pain (e.g. first 24hrs following an operation, managed with analgesia).

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 planned procedures in the project are sub-threshold or of mild (dietary changes or non-invasive imaging) severity (85%). For protocols where the aim is to investigate temporary organ damage, animals will experience moderate severity such as following an operation (15%). In all cases, there will be clear experimental endpoints which are designed to ensure that the animals will only experience these clinical symptoms for a very limited and therefore minimum amount of time.

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?

Our broader project and research program does indeed use non-animal alternatives such as in vitro cell culture and tissue slices for some of the research objectives. However it is currently impossible to accurately recapitulate the entire physiological and pathological responses of organs to injury since there are complex mechanisms involved with multiple cell types (e.g. epithelial, vascular and immune cells), of which we have incomplete knowledge. Therefore for the research aims of this project, it is a necessity to use a minimal number of animals. In the past few decades there has been a great deal of research into advancing mouse models of disease which combined with genetic engineering techniques mean that the mouse is the ideal model for studying these biological phenomena.

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

Alongside the proposed animal work, our research also involves work on primary human tissue and 3D cell cultures (organoids) based in vitro biological models as well as in silico computational modelling. Therefore through the use of these alternative, we will ensure that the animal work is the minimum required and would complement the limitations of the in vitro models. Our intention is that through our in vitro research we will be able to replace some of the animal work in time. In order to avoid any duplication of research and to ensure our methodology is as refined, we will continue our literature searches using public databases e.g. https://pubmed.ncbi.nlm.nih.gov/ and https://www.biorxiv.org/. We also used the following sources of information to search for alternatives to the research proposed: https://www.3rsinfohub.de/organs/organ_liver/index.html https://www.nat-database.org/ https://data.jrc.ec.europa.eu/collection/id-0088

We also used advice published on: https://www.nal.usda.gov/services/literature-searching-animal-usealternatives and https://frame.org.uk/resources/searching-for-alternatives/

Why were they not suitable?

Alternative in vitro models are a hugely valuable adjunct to our research project and we will be able to answer a number of our research questions using these approaches. For example, we will be able to study the effect of genetic changes in single cell types such as epithelial or immune cells. However the in vitro technology is currently inadequate to accurately recreate a complex organ and to recapitulate the multi-cellular contributions to a process such as repair and regeneration which occurs over a period of weeks or months. Therefore there is the need to move to using in vivo animal models to answer the specific questions that in vitro models cannot address.

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 based on our experience and that of collaborators as well as working with statisticians to model the minimum number of animals we will need to ensure statistically robust results. Published data that other researchers have generated was used in order to perform sample size calculations. For each experimental group there will be negative and positive control groups. Variability will be reduced through following standard operating procedures, using the same genetic background of animals for each experiment and through block randomisation and blinding where appropriate.

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 we use as few animals as possible, each experimental design is informed by an extensive literature search to obtain the latest data and to make sure that our work is not duplicating in any way work that has already been performed. This is complemented by small scale pilot studies to determine effect sizes, if necessary. This data together with published data was used to design each experiment, using appropriate power calculations and statistical testing or the NC3Rs' experimental design guidance. Positive and negative control experiments are included in the design and all attempts are made to reducing variability and bias through biological and technical replicates, randomisation and blinding as appropriate. The strategy is analysed and refined as necessary throughout the experiments to identify opportunities to reduce animal numbers whenever possible. An example might be that a result would obviate the need for a subsequent experiment.

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

At all points we will strive to maximise the amount of data generated from each animal. This may involve using multiple tissues for different experiments and sharing these with internal and external collaborators as appropriate. Good colony and breeding management at our facility such as cryopreservation of gametes and embryos will ensure that the minimal number of animals will be used. Wherever possible we will reduce any

variables such as using age and sex matched animals for experiments which will result in more robust results and ultimately fewer animals to 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.

We will study the effects of organ specific gene modifications in mice on development, health and response after injury. Therefore we will use a combination of mice which have been genetically altered (through selective breeding or through modifying individual cells in the same animal) and models of organ injury, both acute and chronic, in order to understand in more depth how organ biology is altered. It is necessary to study both acute and chronic injury since the contributing mechanisms are thought to be very different, according to previous research.

Our proposed methods for making genetic modifications in mice will use the least invasive method possible and so expected to result in the least pain and lasting harm to the animals in order to achieve our scientific objectives (e.g. oral administration of drugs instead of intra-peritoneal wherever possible). The techniques of selective breeding and injections of cells, viruses or chemicals are standardised and with appropriate protocols can be ensured to cause the least possible amount of suffering. Some surgical experiments will be necessary to study the effects of removing parts of organs and with adequate monitoring, analgesia and post operative care, we will ensure the animal does not unduly suffer. We will house the animals in conditions optimised for post-operative recovery and avoid unnecessary stress (e.g. warmer and quieter environments). The duration and end point of each experiment will be the least amount of time required to meet the scientific objectives, which is often after a period of weeks or months which is the biological timescale of organ regeneration.

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

The focus of the proposed project is to study the effects of genetic influences on mechanisms of organ biology in repair, regeneration and ageing. The mouse is the ideal model organism for this proposed work since less sentient animals and genetic models do not recapitulate the tissue heterogeneity and mechanisms of repair and regeneration characteristic of human diseases. There is a wealth of literature and published data demonstrating that mouse physiology, organ injury and regeneration closely models humans. Therefore it is not possible to use more immature life stages which have not fully developed adult organs and nor to use less sentient animals or terminally anaesthetised animals for longer term experiments over several weeks or months.

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

We will establish standard operating procedures, together with experienced technicians and veterinarians, which will ensure appropriate training to recognise signs of potential harm prior to actual harm to animals. We will constantly aim to improve and refine our models and experimental designs to minimise harm including close monitoring of animals after procedures, appropriate anaesthetics and analgesia and non pharmacological measures such as quiet and warm environments. We will use a scoring system, adapted from publications, to set objective thresholds for pain and adverse effects. We will have clearly defined experimental end points which precede humane end points for each procedure and experiment.

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

We will follow the PREPARE guidelines from Norecopa to ensure the highest quality of preparation in the experimental design of any animal experiments. We will be guided by publications such as:

P Workman, EO Aboagye, F Balkwill, A Balmain, G Bruder, DJ Chaplin, JA Double, J Everitt,

DAHFarningham, MJ Glennie, LR Kelland, V Robinson, IJ Stratford, GM Tozer, S Watson, SR Wedge, SAEccles, An ad hoc committee of the National Cancer Research Institute Observers: V Navaratnam and S Ryder, (2010) Guidelines for the welfare and use of animals in cancer research

Smith, A. J., Clutton, R. E., Lilley, E., Hansen, K., & Brattelid, T. (2018). PREPARE: guidelines for planning animal research and testing. Laboratory animals, 52(2), 135–141.

Percie du Sert N, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M, et al. (2020) The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. PLoS Biol 18(7):e3000410.

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

At our establishment we are regularly updated as to the latest news and research from NC3Rs and NORECOPA, for example via newsletters or through relevant conferences. We will also endeavour to apply any improvements or refinements to our protocols during the course of the project.

73. Cell fate during normal and pathological epithelial tissue development

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

epithelial tissue development, stem cells, cancer, tissue regeneration, mammary gland

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 project aims to investigate how stem cells generate the different specialised cell types found in hormonally-regulated organs such as the mammary gland during tissue development and regeneration, and how abnormal regulation of stem cell properties can drive tumour formation in these 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?

In the UK, nearly 1 in 2 people will develop cancer in their lifetime. Breast and prostate cancer are the most common female and male cancers, with around 55,200 and 47,700 new cases diagnosed annually, respectively (Cancer Research UK statistics). Early diagnosis is critical to successful treatment and for increasing a person's chance of survival. It is important therefore to develop better ways of preventing and detecting cancer. Epithelial stem cells are responsible for generating the different specialised cell types required to maintain epithelial organs such as the breast, lung, pancreas by rapidly replacing cells lost through injury or ageing. However, abnormal stem cell behaviour can drive cancer development in several organs. The main goal of this work therefore is to better understand the factors that control stem cells during the development of tissues

such as the breast and prostate, and how they go wrong in cancer in order to contribute to knowledge aimed at improving cancer prevention and early detection.

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

This project will provide new data characterising the dynamics of stem and progenitor cells during normal (including in response to pubertal and pregnancy hormones), and pathological mammary gland development, including tissue injury and cancer development. In addition, this project will assess whether similar mechanisms regulate stem cell dynamics during normal tissue development and also cancer development of related regenerative epithelial tissues, such as the prostate. The knowledge gained in this project over the 5 years promises to contribute to the body of literature and shape future studies focused on developing improved strategies for cancer detection, prevention and treatment in the longer term (>5 years). Results from these studies will be disseminated via presentations at international scientific meetings and publications in peer reviewed journals.

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

Short-term benefits (throughout project):

The data generated by this programme of work will be of value to scientists working to advance understanding of epithelial tissue development and pathology, including tissue injury and tumour development. This includes a better understanding of how hormones control the normal development and functions of these tissues, such as during puberty and pregnancy (mammary only), and how they regulate tissue regeneration in response to injury. Those working on the project will gain experience in cutting edge three dimensional imaging and cell tracking techniques, as well as experimental design and data handling.

Medium-term benefits:

By providing new insights into the impact of age, development and reproductive status (e.g. parity) on stem and progenitor cell behaviour during normal development and in response to tissue injury, and the factors influencing their transformation potential, dissemination of study findings will benefit scientist working both in academia and pharma who are focused on identifying improved methods for the prevention, early diagnosis and treatment of epithelial cancers, including mammary and prostate tumours.

Long-term benefits (greater than 5 years):

Ultimately, the findings are expected to contribute to improved public health by the identification of new risk factors and improved strategies for the prevention and treatment of breast and prostate cancers. A better understanding of how reproductive events and their timing (e.g. of puberty and pregnancy) impact cancer risk will help improve patient stratification and the early detection of cancer.

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

The data generated by the outlined studies will be disseminated via presentations at international scientific meetings, such as the Gordon Research Conference on Mammary Gland Biology and the international ISSCR Stem Cell Meeting, in addition to open-access publications in peer reviewed scientific journals.

In addition, I will seek to develop collaborations with other scientists working in this research field and

other disciplines (e.g. engineering and mathematics), who can use the data generated in our investigations to develop novel approaches to mathematically model stem cell dynamics, or for developing improved image processing techniques. Ultimately this will lead to development of refinements and reduction in animal use. For example, new image processing techniques that allow for better detection of rare or dim cells inside mouse tissues will ensure more data is extracted from every single image which ultimately means fewer images (and therefore mice) are required. Likewise, developing computational mathematical models of stem cell behaviours will mean that experiments can be simulated in silico to test hypotheses, replacing the need for some experiments to be performed in mice. These refinements and new in silico replacement technologies will be published in openaccess publications, as well as making all code open source (e.g. on Github) to facilitate their uptake by scientists working on similar topics and models.

Species and numbers of animals expected to be used

- Mice: 7300

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 primary focus of the outlined research is to identify the biological mechanisms regulating epithelial stem cell fate during normal and pathological organ development and regeneration in mammary tissue. Consequently, the study can only be conducted in a mammalian species as lower organisms do not have mammary glands. In addition, the study requires a species that is amenable to genetic manipulation. Mice are the least neurophysiologically sensitive of the species that meets the aforementioned criteria. In order to enable the full stage of mammary tissue development to be investigated, the study will require the use of mice from embryonic stages through to aged adults and including pregnancy.

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

Most of the animals used in the outlined studies will either be genetically altered or will be administered substances to induce a gene alteration/deletion. The majority of these alteration (>80%) do not have any adverse effects on the animal’s wellbeing and mainly involve changes that enable subsets of cells to be identified. A proportion of the genetic alteration (<20%) are linked to the development of mammary tumours however, animals with these alterations will be carefully monitored and the vast majority killed at an age before which tumours are expected to occur.

Most of the animals used in experimental studies will be given substances by injection using standard routes that cause no more than transient discomfort or pain. Where appropriate, substances will be administered via the diet. None of the substances administered are expected to have any adverse effects.

In order to investigate the regenerative potential of cells, around 50% of the animals used in experiments will be exposed to either a low dose of radiation and/or undergo drug-induced cell ablation. The tissue injury induced is minor and animals undergoing these procedures are expected to continue to show normal behaviour and to thrive. To investigate the regenerative potential of cells in response to physiological tissue regression and remodelling post lactation (involution), some (<20%) animals will be mated

to induce pregnancy and lactation, followed by weaning of litters. This is not expected to have any adverse effects.

For some studies, animals (<10%) will be exposed to a sub-lethal dose of radiation prior to receiving a stem cell transplant. Following radiation treatment, animals may experience a period of sub-optimal health for three to four days, characterised by reduced activity, inappetence and mild weight loss however, thereafter they are expected to revert to normal health and to regain weight.

Approximately 25% of all mice used in experiments will undergo a surgical procedure to implant either a drug delivery device or a small intravital imaging window in the skin overlying the mammary gland. In all cases, surgery will be conducted under general anaesthesia and the animals are expected to make an uneventful recovery and to resume normal behaviour within a few hours. All animals undergoing surgery will be given pain killers upon recovery, which will be maintained until they are showing no detectable signs of pain.

The majority of experiments will last no longer than 3 months. At the end of the study, all animals will be killed using a humane method, to enable their tissues to be harvested either for analysis or for further use in ex-vivo studies.

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

The vast majority of mice used are expected to display either no or only mild clinical signs and are not expected to experience any significant weight loss, overt pain or illhealth. Rarely, some genetic modifications may affect tissues other than our target organs of interest (mammary, prostate) consequently, there is a risk that some mice develop some adverse signs, such as skin abnormalities or weight loss. As it is not always possible to fully predict the nature or severity of any potential defect (e.g. in new GA lines) all new mouse lines will be careful monitoring for possible adverse effects. Should adverse effects arise, that cannot be ameliorated by mild veterinary intervention, the mice will be killed. Some mice will be subjected to higher doses of irradiation which carry a risk of radiationinduced weight loss and a compromised immune system. Animals will either be euthanised prior to appearance of these clinical signs or for longer term studies receive an injection of haematopoietic stem cells within 24 hours of radiation to restore their immune system.

Animals undergoing minor surgical procedures, including pregnant animals, such as implanting small imaging windows or devices under the skin to release medicines slowly, may experience pain and discomfort after surgery. Typically, surgical procedures will entail small skin incisions (e.g. implantation of small imaging windows over mammary tissues or subcutaneous tumours). Thus, mice are expected to recover quickly and will be given post-operative painkillers, which will be maintained until the animals are showing no detectable signs of pain. Implantation of imaging windows during pregnancy carries a small risk (<5%) of abortion or birthing difficulties.

Some mice will develop tumours however, these are not expected to result in suffering as they develop in external tumours (mammary or subcutaneous tumours) and the animals will be killed if the tumour exceed 1.2cm in any direction and the tumours are not expected to metastasise or affect internal organs within the timeframe of the study.

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)?

Breeding and Maintenance of GA mice: 10% mild and 90% sub-threshold

Experimental mice: 75% mild, 25% moderate. This will be dependent on the nature of the genetic alteration, response to cell ablation/injury and whether they undergo a surgical procedure (which carries a moderate severity). The vast majority of animals will make a rapid and unremarkable recovery from the described surgical procedures. <20% of animals may develop tumours (predominantly located in the mammary gland/subcutaneously), which may mean that a subset of these will experience clinical signs of a moderate severity, however the vast majority will not show any overt adverse effects as these are external (i.e. sub-cutaneous) tumours and experiments will be terminated before a metastatic tumour would have time to develop.

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 choice of appropriate species and material to conduct research depends on the extent by which the studied process is conserved across evolution and among different developmental stages and tissues. The proposed project relies on the use of transgenic mice to define the mechanisms of stem cell regulation during development and homeostasis of epithelial tissues such as the mammary gland and prostate in physiological conditions. Organs such as the mammary gland and prostate are very complex structures composed of different cells types (epithelial, immune, stromal cells) that communicate with each other during organ development, maintenance and repair. These complex and highly dynamic interactions cannot be replicated using the in vitro systems currently available. By its very definition, the mammary gland is specific to mammals, making the mouse the best-placed lower organism to conduct our studies. Similarly, the mouse is currently the best available system for studying prostate development. In addition, transgenic mice currently represent the best model system for studying cancer and its biological basis. Ex vivo tissue culture using human tissues are limited by the availability of some tissues, and the challenges of maintaining them long term outside the body, meaning they are only suitable for acute, short term (< 7 days) studies.

Thus, notwithstanding our efforts towards using in vitro systems (e.g. organotypic cultures) that replace the use of animals where possible and appropriate, we will critically require to perform some experiments in mice, in order to visualise epithelial tissue development in their natural and physiological microenvironment. The rodent model is the least severe approach to addressing our objectives, and are therefore necessary to complete the goals of the proposed research.

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

Where appropriate we will use *in vitro* models of immortalised primary cell lines and 3D organoid cultures to undertake more detailed functional and molecular analyses of individual pathways and genes, in addition for screening chemical compounds and/or

growth factors that are considered candidate effectors of epithelial stem cell behaviours. Time-lapse imaging of 3D organoid cultures will also be used where appropriate (e.g. when we don't require the presence of other cell types in the microenvironment) to support the proposed intravital imaging experiments, which will ensure that fewer mice undergo this technique.

My lab is also focused on undertaking genetic epidemiology analyses of human populations to investigate the causal impact of developmental exposures on cancer risk, particularly breast and prostate cancer. Our recent collaborations with genetic epidemiologists have generated data and hypotheses that can be tested in the laboratory using human primary cells and tissues where available. This will help to reduce the number of mice used during this project, as we will bypass mouse models completely to investigate promising pathways and targets identified by genetic epidemiology approaches in human tissues only. We are currently seeking ethical approval to collect human breast samples from a local hospital which will help reduce animal numbers.

Why were they not suitable?

Epithelial organs such as the mammary and prostate glands are complex tissues comprised of multiple cell types that undergo phases of rapid growth and regeneration. It is not possible to mimic this completely in culture. *In vitro* models do not recapitulate the complex physiology of tissue and disease development, as they do not include the full spectrum of different cells that may a role in these processes. Particularly, current *in vitro* techniques cannot model the complex and fluctuating systemic

hormonal environment crucial for regulating prostate and mammary gland development and tumourigenesis. However, I have developed a 3-dimensional organoid model, as well as a tissue explant culture system of the mammary gland during the previous project timeframe, that can be used to replace some of the animals that would otherwise be required in this project. In addition, as described above, we will rely on human primary cells and tissue where available and appropriate.

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 project will require complex genetic crosses. We will design the mating protocols to maximise the number of mice with the correct genotype and will keep the number of mice as low as possible in initial crosses until the desired genetic combinations are achieved. However, some strains may be unable to be maintained as homozygotes, meaning that these breeding strategies are less efficient that with homozygous strains. Based on my experience of complex genetic crosses encompassing a number of alleles, including the models developed over the current project, as well as the requirement for female mice for the majority of studies, breeding is estimated at approximately 750 animals per year. Mice of the incorrect genotype will be used as controls or replacement breeders, which will not be as heavily reliant on having all the alleles required to address the other objectives of the project.

Experimental usage is estimated at ~450 per year, which is around 40 experiments per year divided between 4 researchers, based on our experience in the experiments undertaken in previous project with 1 researcher. This is based on advice from our in house statistician, and using calculations including typical variations observed in our experiments and in the literature. Overall, our calculations indicate we need group sizes of 10-12 to achieve statistically robust results.Detailed justification of mouse numbers is provided within each protocol. As our group grows and if more funding is acquired we may perform experiments in different strains (e.g. different tumour models, new GA mice based on results of RNA-seq experiments etc), which will mean that these numbers will need to be adjusted accordingly.

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

In addition to seeking the advice of a local statistician, online resources were consulted including the NC3Rs' Experimental Design Assistant (EDA) which helps guide experimental design and appropriate statistical analysis, as well as guidance on blinding and randomisation. We use online tools such as G*power to perform calculations as advised by the in house statistician

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 strategies throughout and pilot studies will be performed where appropriate to ensure that the minimal numbers of animals are used during the course of this project. At experimental endpoints, as many tissues as possible will be harvested and made available to other researchers working on related research questions. Tissue sharing is facilitated by the Institution's (The Primary Establishment) Internal Tissue Sharing Network. The Primary Establishment are currently developing the "Tried & Tested: The 3Rs Open Lab Book" which will be a searchable 3Rs compilation resource built by the animal research community at the Institution to share 3Rs successes, tips and also details of when experiments could be further optimised. This will help reduce the number of pilot studies required, ultimately reducing 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 the mouse as our model during the course of this project. Our work requires modulation of specific genes and the mouse is the only model that we can use to carry out genetic studies in epithelial tissues such as the mammary gland and prostate (which are mammalian specific organs). Inducible conditional gene targeting will be used to delete the gene under study only in the tissue of interest (e.g. mammary gland only) wherever possible. This will provide better experimental data, in addition to reducing any adverse effects from deleting the gene in other tissues, thus representing a refinement.

Most of the protocols are in the mild category and animal suffering will be minimal. For longitudinal intravital imaging studies, only animals destined for imaging will have the intravital imaging window implanted, and mice will be killed immediately if images of sufficient quality cannot be obtained.

In mammary gland and sub-cutaneous xenograft tumour studies, the size of tumours may be up to 1.2 cm at the widest point. Post-mortem assessment of tumour growth in our pilot studies, however, will be used to refine end points further.

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

Non-mammalian animals are limited in their use as they lack the epithelial organs of interest in our research (e.g. mammary gland, prostate). Moreover, mammary gland and prostate development and tumourigenesis mostly occurs postnatally. Thus, we will perform most of our studies in animals older than 21 days old. However, some studies (e.g. regarding the role of stem/progenitor cells in the first branching event in these organs) will be performed in embryos where appropriate. For studies involving long-term time-lapse intravital imaging, animals will not be allowed to recover and will be killed at the end using a schedule 1 method while still under anaesthesia.

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

Data generated in the previous project has enabled us to refine some procedures, including radiation dosages. Pilot studies will be performed to refine doses of administered substances and tumour endpoint sizes, which will also provide valuable statistical data that can be used to refine cohort sizes in future experiments. Post-mortem assessment of tumour growth in our pilot studies will also be used to refine end points further.

We will continue to refine our methods for monitoring animal discomfort after surgeries and clinical disease in tumour models. This includes an analgesic-anaesthesia management plan in accordance with the local named veterinary surgeon (NVS). We will use general anaesthesia and administration of analgesics to all procedures considered more traumatic for the animal than the introduction of a needle. This includes cases where only very small incision sites (<1cm) will be performed. We use a general gaseous isoflurane anaesthesia carried by a mixture of oxygen and compressed air. When this is not possible, we use a general anaesthesia by injection, using a mixture of xylazine/ketamine/flunitrazepam as per NVS guidelines. During the time of the procedure requiring anaesthesia, mice will be kept on a heating pad in order to maintain the temperature of its body. After surgeries, animals will be maintained on the heat pad or in temperature regulated recovery chambers until they wake up. Continued analgesics will be provided in days after surgeries where required and in accordance with the NVS recommendations. Specialised bedding and enrichment suitable for postsurgery recovery will be provided to animals undergoing any surgical procedures.

All animals will be assessed daily for signs of adverse effects. However, monitoring regimes and frequency will be adapted as necessary to be proportionate to anticipated/observed effects, e.g. use of scoresheets if adverse effects are noted and when mice possess tumours. All mice will be monitored in accordance with the NC3Rs Welfare Assessment Guidelines.

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

We will participate in 3Rs seminars, presenting our approaches and learning from others. We will keep abreast of the PREPARE guidelines, as well as updated guidance and publications from the NC3Rs and LASA.

Mammary gland related protocols are published as methods books, which are often updated to include more recent techniques and refinements. The more recent edition was published in 2022 during the previous project and we will refer to these publications where relevant. In addition, researchers are increasingly publishing refined protocols as videos online (e.g. JOVE) which we will also use to keep ourselves updated. In addition, I routinely attend the annual Mammary gland methods workshop run by the European Network of Breast Development and Cancer Labs (of which I am a member), where recent technical advances in the field are publicised, including refinements to standard mammary gland specific protocols.

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

By continually keeping up to date using resources online (e.g. NC3Rs website and regular newsletters), following related accounts on Twitter and LinkedIn and by participating in local, national and international meetings focused on animal research such as the Regional 3Rs symposia. The 'Tried & Tested: The Bristol 3Rs Open Lab Book' currently underdevelopment will be searchable 3Rs compilation resource built by the animal research community at the University of Bristol to share 3Rs advances and successes, and guidance on how to implement these advances in the our own laboratory.

74. Intrinsic & extrinsic effects on B cell differentiation

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

B cell, Immune response, autoimmune response, ageing

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?

B cells are the cells that produce antibodies after vaccination. We plan to study B cell activating factors - signals that are transmitted through surface receptors on B cells or are delivered by cells that interact with B cells.

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 bodies can defend themselves from infections by producing antibodies. Antibodies are proteins that recognise and bind specifically to bacteria or viruses (pathogens). This then leads to the destruction of these pathogens by the rest of the immune system. Antibodies form after infection by a pathogen and can give life long protection from that specific pathogen. The result will be the generation of specific antibodies and long term immunity. Antibodies are produced by B cells. The purpose of this project is to understand how B cells are activated by pathogens, how this makes B cells develop antibodies that are highly specific for a pathogen, and how the B cells transform into cells that make huge amount of antibodies, or how B cells transform into memory B cells that survive for a very long time protecting us from illness for many years.

The Covid pandemic was a reminder how important vaccination and the rapid design and generation of vaccines specific to new threats is. Vaccination is a way of mimicking infection by a pathogen without the danger of developing an illness from that pathogen. Vaccination leads to the development of antibodies in exactly the same way to what happens during natural infection by pathogens. Because the fundamental processes how B cells develop and how they interact with other immune cells are not well understood, it is still difficult to design and generate vaccines in a targeted way. Understanding these processes better may lead to more intelligent ways to generate vaccines that induce protective B cell responses.

During ageing our body reacts far less efficiently to vaccination or produces antibodies in response to infection. Understanding the defects that inhibit responses in the aged will help create treatments that may enhance the aged antibody response.

Antibodies not only protect, but they can also generate illness. Some people develop autoimmune disease where B cells start making antibodies to structures in our own bodies. How B cells are triggered to make antibodies to ourselves is another part of this project. Understanding this may lead to new ways how to treat or prevent autoimmune diseases.

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

After vaccination, B cells can fine tune the specificity of antibodies they generate towards pathogens. The project will create information on the fundamentals how this process works, how cells interact to regulate the process, and what kind of signals are exchanged. We will create scientific publications about our results. Further, we will publish and discuss our data with others working in the field on scientific conferences. This will benefit others working on understanding vaccination or the antibody defence to pathogens to better understand these processes.

B cells themselves can also cause disease by inappropriately getting activated to react to our own bodies. This is called autoimmunity. Understanding the factors that activate B cells to vaccines will also help understanding inappropriate activation of B cells triggering autoimmunity. By the end of this project we plan to have published scientific data on this. This work will facilitate the work of others studying autoimmunity, how to prevent autoimmunity being induced, or how to cure autoimmunity.

In the longer term this work should lead to knowledge how to better induce antibodies in animals, for example during the generation of new antibodies that can be used as human drugs. It should lead to better ways to generate human vaccines to pathogens or enhance antibody responses to vaccination or infection in aged people. Understanding autoimmune processes should lead to the development of drugs that can prevent autoimmunity or cure autoimmunity.

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

Fundamental research

Scientists studying B cells, antibody responses, vaccination, and the immune response to pathogens will directly benefit from this work in the short term.

Biotechnology

Scientists developing vaccines to pathogens, monoclonal antibody drugs targeting a huge range of diseases, or drugs directly targeting autoimmune disease should benefit from information that will enable their work in the medium term.

Patients

In the long term, patients may benefit from improved vaccines or vaccination schedules, drugs enhancing antibody responses to infection or in aged patients, and drugs targeting autoimmunity.

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

The output of this project will be presented and discussed at national and international conferences. Outputs will be published in high profile open access journals. The public and peers will be informed about these by press releases and social media. Manuscripts for publication will be prepared according to ARRIVE guidelines.

We collaborate with clinicians who work on infection, aged patients, or patients with autoimmune disease. We collaborate with industry who develop genetically engineered mice to facilitate the rapid generation of human antibody drugs.

Species and numbers of animals expected to be used

- Mice: 35,720

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 adult mice, because they are the only well studied mammal that has an immune system that is sufficiently similar to the human immune system. Lower vertebrates than mammals lack many of the features of the immune system we share with mice and other mammals. Only in mice experimental methods have been developed that will allow us to undertake our work.

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

Typically, mice will experience mild, transient pain and no lasting harm from immunisation. Immunisation will be done by injection using standard routes or injection into the foot. Foot immunisation will be done under anaesthesia and may lead to short-termed mild swelling that will not affect normal behaviour. Mice may be reimmunised or injected with immunomodulatory substances. No animal will be injected more than four times. Blood samples will be taken at the beginning of the experiments, before and after immunisation. Most animals will be killed within 10 weeks of immunisation. The test substances will already have been tested to ensure that the dosing regimen does not cause toxicity. The final procedures 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.

When we use infectious agents that replicate, we will prioritise the use of attenuated bacterial or viral strains, 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.

Some animals will be treated to induce autoimmune responses. In a model for arthritis, mice will be injected with agents that induce antibodies that can lead to arthritis. In a model for Sjogren's disease, fluid containing a virus will be delivered through the salivary gland duct under anaesthesia. As these experiments are performed to study the induction of antibody responses, most animals will only be maintained until the autoimmune antibody response has formed and most experiments will be terminated before autoimmune disease can develop.

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

For most of the mice, including immunodeficient strains, we do not expect any impacts or adverse effects in our AAALAC accredited specific pathogen-free (SPF) animal care facility.

Embryo transfer and vasectomy are surgical procedures with short term post-surgical pain. Postsurgical pain will be controlled by analgesia and any animal not fully recovered (eating, drinking, return to normal behaviour) within 24 hours will be euthanised.

The adverse effects of immunisation/infection or immunomodulation include systemic or specific tissue inflammation which will be transient, lasting for a few days. In the case of influenza virus there will be substantial weight loss which is restored within two weeks. Appetite stimulants are fed in mash in addition to normal diet during the course of the experiment to try and ameliorate the percentage of weight loss.

Some viruses will induce chronic infections with adverse effects such as weight loss and while this may be moderate in C57BL/6 other inbred strains manifest enhanced vascular permeability, lung immunopathology and animals will be closely monitored according to the humane endpoints detailed.

Foot immunisation, done under anaesthetic will often lead to foot swelling that can last a week, but does not affect normal behaviour.

Although ageing is a major risk factor for adverse effects, we know that the vast majority of our aged mice remain healthy throughout the duration of their lifetime. There is an increased incidence of adverse effects not observed in young wild-type mice including altered coat condition, diarrhoea, eye abnormalities, abdominal distension, movement issues, tremors and seizures. A tiny minority of these develop tumours, but regular checking by our experienced animal technicians ensures these are detected early, and the mouse euthanised immediately. A specific code of practice for caring for aged mice is in place.

All forms of arthritis cause joint stiffness and some degree of disability for the duration of the study (100% incidence). Arthritis will be monitored carefully using a scoring system that monitors and scores behaviour; coat condition; body weight; mobility and weight bearing; grimace and calliper measurements of the injected joints. Measures will be taken to ameliorate these adverse effects eg soft bedding on the cage floor.

Mice will have salivary gland cannulation under the anaesthesia, which may cause stress, and slight swelling of the salivary glands post cannulation. The swelling usually resolves in 48-72 hours.

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 for most of the mice will be mild or sub threshold

Total animals used = 35,720

Mild: 37% (13,070)

Moderate: 5% (1,750)

Sub threshold: 59% (20,900)

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 the animal model to understand how antibodies are generated in response to vaccination. This process happens in lymphoid tissue such as spleens or lymph nodes. Lymphoid tissues are very complex structures with many different cells types interacting and communicating with each other, and cells migrating through different sub-compartments that provide different environment with different interacting cells and stimuli. These processes are so complex that currently no in vitro system is able to replicate this.

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

We are collaborating with physicists that computer model immune responses.

In vitro models, where cells interact in test tubes in solutions outside the body are generally not sufficiently complex to recreate the complex sub-compartments in lymph nodes.

We are currently working on modeling the interactions happening between immune cells using proteins or stromal cells in the test tube (in vitro) in the lab.

Why were they not suitable?

Whilst computer modeling of immune responses is ongoing and improving these do not yet begin to replicate the complicated conditions and variables that exist in the immune system. Our use of computer models helps to confirm some of our data and provide testable hypotheses but is limited in scope and output to small aspects of the B cell response in vivo. While reducing animal experiments, computer modeling typically creates new hypotheses that have to be tested in the intact organism.

In vitro models can be useful to replicate and study processes during B cell stimulation in the lab. They are used in our laboratory when appropriate, but do not replicate the complexities of the environment in which immune responses occur.

Similar to computer modeling or modeling of immune responses using proteins or stromal cells in the test tube (in vitro) can reduce the number of experiments, but also creates new hypotheses that have to be tested in the living organism.

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 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. Sample sizes for our experiments are estimated from past experiments, with power calculations done with support from the local statistician at our Institute using exemplary data from earlier studies

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

Advice from local professional statisticians has been sought to evaluate proposed experiments for statistical validity and in the generation of power calculations. Numbers of animals necessary were calculated using our own historical data to estimated the expected variability of our experiments and to calculate the minimal number of animals necessary to generate significant results.

Further, we are continuously refining our analytical methods and use the NC3R's Experimental Design Assistant to ensure we are considering all relevant aspects of design, in order to reduce variability, allowing further reduction in animal numbers. All relevant tissues where possible will be frozen and stored as input and controls for downstream experiments. Shared use of these will be offered for shared use by other groups working on related questions.

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 minimise use of animals by keeping colony sizes as small as possible. This may lead to experiments having to be split into two independent repeat experiments to generate sufficient power. New substances used on animals will be tested first in small pilot studies. Computer modeling may be used to predict experimental conditions that will show the largest effect sizes. At the end of the experiment, we will harvest the maximal

possible number of tissues. Tissues not immediately analysed will be archived in frozen state and will be made 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.

Some animals will be allowed to age to study the immune response in the ageing organism.

In order to induce gene expression in animals or deplete specific cells some animals will have receive substances via gavage (force-feeding), injection, or through food. Oral gavage or injection can be necessary to induce a rapid onset of gene expression. Use of flexible plastic gavage tubes over metal fixed cannula will reduce risk of trauma and inflammation. This will allow us to study processes that happen within short time periods of a few hours.

We will have to induce immune responses to study the response to vaccination. Animals will be vaccinated using methods similar to human vaccination, e.g. injection of substances under the skin, intramuscular injection or by intraperitoneal or intravenous injection.

Some animals will have to be vaccinated into the foot, as this induces a strong response in local lymph nodes. This will be done under short term anaesthesia; however, the animals will suffer from temporary swollen foot for several days. This has not led to changes in normal behaviour in the past indicating that there is no major discomfort. If animals show signs of significant foot swelling or inflammation, they will be treated with analgesic agents.

Why can’t you use animals that are less sentient?

Non-mammalian animals are limited in their use because they either do not possess B lymphocytes or their differentiation in response to stimulation is too removed from the human immune system to provide relevant results. Embryos are unsuitable as their immune system is not mature and does not 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?

Ageing animals will be carefully monitored by trained staff 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. 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 with that may develop severe effects will be killed humanely.

If gene induction or deletion is done for a new gene animals will be monitored closely in the days after induction. Mice will be weighed daily to detect weigh loss. If any mice have reduced activity, ruffled coat or hunched appearance they will be warmed and given glucose-saline (subcutaneous injection) to reduce heat loss and dehydration which may be a contributor. Flexible gavage tubes dipped in sucrose will be used to minimise damage to the oesophagus. Refined mouse handling technique and technical expertise will minimise any discomfort. Time and route of induction will be optimised in preliminary experiments for efficient induction or deletion of transgenes using the least adverse route of administration.

For all new models, new methods and new antigens we will consult with expert staff at our animal facility. Some methods, e.g. animals that are receiving new antigens or immunisation protocols, will be carefully monitored during the protocol and humane end points will be used if necessary in consultation with expert staff at the animal facility.

Immunisation will be done via injection of antigen. These vaccinations should only have transient effects and animals should return to normal behaviour within two hours. If new antigens will be tested, this will be discussed with expert staff at the animal unit. New antigens will be tested on small groups of animals first.

Some animals are immunised in the foot under short term anaesthesia. Foot immunisation may lead to foot swelling due to inflammation. This should not be strong enough to lead to behavioural changes. Mice that do show excessive inflammation or lameness will be treated with analgesics. Refined mouse handling technique and technical expertise will minimise any discomfort. Mice are not re-injected until fully recovered from previous injection and never at a frequently that causes them to display anything other than transient pain to discomfort. Foot immunisation has been refined by injecting substances under the plantar surface of the foot, away from the weight bearing walking pads. Further, injection of non-immunogenic substances will be done by injection into the hock above the foot.

LASA guidelines will be followed regarding volume of substances to be administered.

Pathogens used are weakened or killed versions that should be non-pathogenic within the time-scale of the experiment. When pathogens are used, animals will be monitored appropriately. In the case of bacterial infection mice will be monitored for the first 48 hours following immunisation and also during the third week of infection which is the time of high susceptibility of secondary infection. The dose of bacteria administered is the lowest possible to obtain a response. Expert animal handling staff are aware of the course of the infection and adverse effects are expected to be noticed and dealt with quickly.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Experiments will be performed in line with LASA guidelines, and using the NC3Rs Experimental design tool.

Will ensure that all experiments are designed to allow reporting in accordance with the ARRIVE guidelines. Our research will be published according to ARRIVE guidelines, regardless of whether a journal endorses this.

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, have signed up to the NC3R newsletter, will use the regular NIO newsletters containing latest 3rs advances and opportunities, and attend Regional 3Rs symposia.

75. Mechanisms of signalling and cell-to-cell communication in infection and inflammation

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

RNA communication, cell-to-cell communication, Gastrointestinal nematodes, extracellular RNA, vaccine

Animal types	Life stages
Rats	Adult
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 overall aim of the project is to understand how cells use and deliver molecules to communicate with one another during health conditions and disease conditions. Pathogens often release ribonucleic acids (RNAs) that instruct host cells to change their properties so as to benefit the infection. In order to determine the different ways that pathogens can use RNA during infections we examine how RNAs travel from one cell to another. We aim to determine whether and how there is specificity in which cells receive and respond to extracellular RNAs in the body. We will use genetic tools and vaccination strategies to block RNA communication and test its importance in viral and parasite infections. Finally we will use synthetic reagents to mimic or inhibit RNAs that are transmitted between cells or between pathogen and host in order to understand their importance during infections and their capacity as signalling molecules.

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 on extracellular RNA will help advance the understanding of immune signalling and cellto-cell communication that is of relevance to many investigators nationally and internationally, spanning many disease models.

Longer term: This work may identify new molecules and mechanisms that promote or inhibit inflammation and may help move forward new vaccine candidates for gastrointestinal parasites that are urgently required in the developing world (e.g. hookworm infections) and livestock. Our work may also inform the mechanisms by which viruses and other pathogens influence immune cells and pave the way for new antivirals to disable viral or host molecules required during infections. The basic research on new mechanisms of cell-to-cell communication could also hold relevance in cancer treatments.

What outputs do you think you will see at the end of this project?

Intended outputs: A challenge in the field of extracellular RNA research is the lack of knowledge on how RNAs are exported by donor cells and how these can target specific recipient cells and function inside them. Our basic research will identify the factors (proteins) required for RNA trafficking and build a quantitative model for how imported RNA regulates the functions of immune cells. This will provide new biochemical understanding on intercellular communication and identify new targets in infectious and inflammatory disease. We will modulate the expression levels of genes involved in RNA trafficking in order to characterize the importance of this communication mechanism in models of virus and parasite infection and inflammatory disease.

Who or what will benefit from these outputs, and how?

In the next 5 years our research on molecular mechanisms by which extracellular RNA operates will help advance biochemical understanding of intercellular communication that is of relevance to many investigators nationally and internationally, spanning many disease models. Our work on helminths may lead to new vaccine development for hookworm infection, which is urgently required in the developing world and new vaccine development in livestock infections needed for food security and economic viability in the UK and worldwide. In the next 5-10 years, this work may advance new antiviral therapeutic strategies targeting extracellular RNA which would benefit global health.

How will you look to maximise the outputs of this work?

By publishing our work and presenting it at international conferences we will inform the wider scientific community of our results. This impacts national and international academics spanning the fields of immunology, parasitology, virology and extracellular vesicle (EV) biology and RNA biology.

We will also continue to engage with Industry to develop the path to advance our basic discoveries in RNA towards new drugs (e.g. antivirals) or vaccines (e.g. for hookworm) to benefit global health. We will also engage with policy makers on the opportunities to use new knowledge and technologies for societal benefit in medicine and agriculture.

Species and numbers of animals expected to be used

- Mice: 11000
- Rats: 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.

In order to achieve our objectives we need to generate and breed Genetically altered animals (and associated WT controls) which have alterations (e.g. knockout and knockin tags) in the mouse genes of interest. “Genes of interest” are those that are associated with RNA transmission and these are determined through our in vitro analyses (immunoprecipitations of RNA-protein complexes, proteomics) and by reference to the literature.

Adult mice will be used in the project with the exception of breeding and maintenance which will use all life stages.

Typically, what will be done to an animal used in your project?

Animals will be housed in single sex groups of no more than 6 per cage, with a continuous supply of food and water as well as cage enrichment as standard (e.g. play tunnels, chewsticks).

Typically animals may experience brief, slight discomfort and no lasting harm from the administration of substances via standard routes (orally, subcutaneous, intravenous, intraperitoneal). The route and frequency will be appropriate to the drug and purpose, but typically no more than one injection daily.

Body fluids may be taken using a combination of volumes, routes, and frequencies that of themselves will result in no more than transient discomfort and no lasting harm.

Animals undergoing Irradiation and BM transfer will be irradiated (whole or partial body) with a single or split dose followed by administration of BM cells in order to track and capture the specific transfer of tagged RNA and protein complexes or test the function of deleting an RNA or protein that is trafficked into a cell. These will be tested first in vitro for toxicity and animals will be closely monitored and any animals exceeding moderate clinical signs will be humanely culled.

Animals may experience brief, slight discomfort and no lasting harm after Helminth, Viral or bacterial infection. Infections will be administered orally, subcutaneous, intravenous, intraperitoneal, intranasally or as an admixture with drinking water.

Volumes and frequencies of administration will be in accordance with recommended maximum volume limits outlined in LASA good practice guidelines.

At the end of study, the animal may be Killed, the duration of experiments will vary.

What are the expected impacts and/or adverse effects for the animals during your project?

Helminth infection is in general well tolerated and does not cause mortality or systemic pathology. Momentary discomfort from inoculation will be minimised by using good

technique. Bacterial infection with fully attenuated microorganisms of animals, including if previously treated with antibiotics, may cause minimal symptoms such as slight loss of coat condition but symptoms rarely exceed temporary reduction in activity and hunched position, and animals make a full recovery.

Bacterial infections (e.g. Listeria monocytogenes) or viral infections (e.g. Influenza) may cause symptoms of moderate severity but these infection models are well established. Any animals displaying symptoms of ill health such as shivering, piloerection and weight loss will be closely monitored as to not exceed the moderate severity limit.

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 actual severity limit for most (~70%) mice will be subthreshold or mild. The majority of animals are bred and used to supply tissue for in vitro analysis. The breeding of the majority of transgenic lines is listed as mild, and the majority have no defects other than compromised immune systems and suffer no ill effects in our clean animal facility, therefore the actual severity returned is subthreshold. In our experience, we expect that most of the genetic manipulations in this project licence will not result in an adverse effect on the welfare of the animals. The impairments can generally only be detected either by phenotypic analysis of their haematopoietic system or after experimental immune challenge in vitro or in vivo.

For pathogen infection, most of our studies involve the parasite Heligmosomoides bakeri which is a natural pathogens of mouse that does not have adverse effects on its host in the lab and so most of the animals undergoing these procedures, even with immunological manipulation, will typically not exhibit any discomfort. A small number of our studies (<10%) will involve other pathogens including Influenza where the severity limit may reach a moderate level. This is necessary as our project aims at defining the underlying mechanisms of pathological infection situations.

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?

Our aim is to understand the basic mechanism and function of extracellular RNA in immune signalling and infection. Only with this information can we design new strategies to control pathogens and the inflammation that they cause. Many aspects of immune signaling require an intact physiological system. At the same time, we are continuously making efforts to find alternatives to animal use, which is why as much as possible our proposal uses in vitro approaches and ex vivo analyses of cells as well as organoid models to achieve our goal of understanding how RNA is transmitted to cells and how this impacts gene expression and organ (epithelium) function. We also work with

computational biologists to develop strategies to predict interactions in silico and steer our validation experiments to only those interactions where there is strong support for function (for example based on conservation across pathogen species).

Which non-animal alternatives did you consider for use in this project?

Certain functions of the immune system and the functions of specific cells upon treatment with pathogens can be reproduced to an extent in vitro and some insights into RNA transmission can be gained from experiments using immortalised cell lines.

Where possible we will also carry out genetic manipulation in organoids and primary cells in vitro, which will further replace the necessity of producing genetically altered mice in some instances. For example if we identify specific candidate proteins that are important for import of RNA to cells we can first test their phenotypic functions in the context of the epithelium using organoids to reduce the number candidate proteins to be tested in mice.

Why were they not suitable?

Information from these sources is limited for a number of reasons. First, immortalised cell lines are by their nature different to primary cells as the act of immortalisation activates oncogenes which promote cell survival in vitro. These changes can have profound effects on signal transduction and RNA transmission which is the primary focus of the proposed study and therefore results obtained with such cell lines will not achieve our objectives.

Second, many aspects of signaling can only be studied in vivo where the microenvironment and spacial organization of cells impact the mechanisms of RNA transmission and the cell types that participate. Third, responses to pathogens and resolution of inflammation can only be faithfully reproduced in an in vivo setting as these require precise interactions of multiple cell types in distinct environments and migration of cells into and out of specific organs. Fourth, to understand immunosuppression by the pathogen (and the role of RNA in this) we must be able to assess the intact immune system rather than isolated components of that system in vitro.

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 numbers based on our previous usage in the last 5 years and refined research goals for the next 5 years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our experiments are designed to minimize the numbers of animals required and based on previous experiments we expect that outputs will be measurable, reproducible and statistically significant typically with group sizes of 4-8 mice for in vivo experiments. We have a designated statistician as part of the Institute of Immunology and Infection Research, who is asked for advice on experimental design on the occasions when we are uncertain of which approach to take. We use ANOVA to combine results from repeat

experiments to increase statistical power. If required we can increase the number of mice in the repeat experiment based on the results of the initial experiment. Alternatively we can perform additional repeats to increase samples sizes if required. This allows us to minimize animal usage as we can start with smaller group sizes and incrementally increase them as required.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Development of in vitro techniques such as gene knockout in organoids also reduces the numbers of animals required for the project. In this manner we may be able to test initially the functions of multiple genetic alterations in an in vitro tissue model without having to breed together individual mouse strains harbouring the mutations thus greatly reducing our mouse breeding. The number of animals used to generate parasite material is kept to a minimum to provide only the essential level of biological samples consistent with the research program. Overall, the numbers of mice used will be kept to an optimal minimum that aims on one hand to generate reliable results and on the other to contribute to the Reduction aspect of the 3R's. The numbers will be kept as low as possible by good experimental practice that reduces the need for multiple repeats of experiments, by vigilance of the breeding program, by information gathered continually from our activities, by pilot studies that will indicate the optimum strategy to follow in order to answer the objectives' questions and by the judicious use of controls within the experiment.

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 order to achieve our goals as set out in the Objectives we propose to use the mouse as the model organism to study RNA transmission and its role in immune signaling for several reasons. Mouse transgenic and knockout techniques are well established; mice have a relatively short generation time; mouse haematopoietic system has been extensively studied and, in addition to the accumulated knowledge, there exists a vast array of reagents that facilitate the studies to a level unknown for many other organisms.

For pathogen infection, most of our studies involve the parasite H.bakeri which is a natural pathogen of mouse that does not have adverse effects on its host in the lab and so most of the animals undergoing these procedures, even with immunological manipulation, will typically not exhibit any discomfort. A small number of our studies (<10%) will involve other pathogens including Influenza where the severity limit may reach a moderate level. This is necessary as our project aims at defining the underlying mechanisms of pathological infection situations. Without inducing and analysing such conditions we will be unable to generate reliable data that would answer the questions posed by the objectives of this programme. Animals will be monitored for clinical symptoms such as weight loss and signs of ill health in order to follow the progress of the animal and allow intervention to terminate humanely the experiment in case the level of severity exceeds that set by the protocol.

Why can’t you use animals that are less sentient?

Mice provide an important model for virus infection and helminth infection, which cause pathogenesis similar to that found in humans. To our knowledge no other species of lesser sentience can fulfil the requirements of this project to the same extent as the laboratory mouse.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

My group will work closely with Bioresearch & Veterinary Services to ensure we are following current best practices. We will utilize the comprehensive guidelines and standard operating procedures for most common rodent procedures. Animals will be provided with environment enrichment as standard by Bioresearch & Veterinary Services facilities. Anaesthesia and analgesia will be provided where suitable. Immunocompromised mice will be housed in individually ventilated cages.

Standard Operating Procedures will be utilized to minimise transient pain, or early endpoints will be used that prevent animals experiencing more severe harms.

We will ensure that the lowest doses of agents are used, along with the most appropriate method of delivery, to induce meaningful and measurable infections and/or immune responses while reducing the harm to which animals are exposed

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will consult published Guidelines provided by the National Center for the Replacement Refining & Reduction of Animal Research (NC3Rs) to ensure we are following current best practices.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed about any advances or developments in best practice and methods by ensuring our attendance at seminars or workshops on the 3R's as well as maintaining constant communication with Bioresearch & Veterinary Services. We will also consult the National Center for the Replacement Refining & Reduction of Animal Research (NC3Rs) website regularly.

76. Analysis of fish nervous system development in health and disease.

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

zebrafish, brain, eye, behaviour, neurodevelopment

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?

We seek to understand how cells organise to form organs such as the eye and nerve cells connect to form the central nervous system and how the brain receives and processes information from its environment to evoke behavioural responses.

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 further understanding of how genetic mutations, gene interactions and the environment affect tissue formation, cell and circuit development in the brain and retina. This research advances our knowledge of nervous system formation and bridges the gap between discovery research in model systems and human disease phenotypes. We will improve our understanding of normal eye and brain development and will use new zebrafish models of human eye and brain diseases to gain further insights into the causes of hereditary ocular malformations and brain conditions such as autism, schizophrenia, depression, dyslexia and early childhood diseases. Our research has the potential to benefit human health as our data and disease models can be used in drug development and improved diagnostics.

What outputs do you think you will see at the end of this project?

Our comprehensive analyses of the genes and genetic interactions that build the eyes will provide a wealth of information of value in the diagnosis and understanding of debilitating congenital abnormalities of eye formation. Our current project focusses on AMC phenotypes (Anophthalmia (absence of eyes), Microphthalmia (small eyes) and Coloboma (failure of optic fissure fusion; AMC), highly variable congenital conditions that severely impair vision in humans. Our research will complement, and both inform and be informed by large scale genomics studies in humans (including UK Biobank and 100K Genome projects). These projects will identify many AMC candidate genes, the fish orthologues of which can be functionally assessed through our studies.

Our brain asymmetry project will contribute to a broader understanding of the developmental basis and functional consequences of forebrain asymmetry. Brain asymmetries are of significant interest to neuroscientists and neurologists given roles in depression, anxiety, addiction and decision-making. We will also pioneer the use of multi-modal data analyses to link circuit composition to behavioural outcome. Our innovative genetic screen will generate a wealth of knowledge on novel gene functions and genetic interactions that will be an important resource for the community.

We also use zebrafish to model some aspects of early childhood diseases that affect metabolism in tissues and cells. In humans, changes in the concentration of metals, such as manganese for instance, leads to early onset Parkinson's disease affecting movement and cognitive functions. We are trying to find new drugs for treatment with reduced side-effects compared to current treatment options.

Our research will be disseminated in peer reviewed publications and communicated at scientific conferences. We also have a website where we publicise our research and outreach work. We provide an outstanding training environment for our researchers and others who engage with the project, from school students to senior scientists.

In addition to scientific knowledge, our targeted genetic screen and deep phenotyping platform will generate many tools and resources including;

New lines of zebrafish carrying mutations and/or transgenes

Phenotype information (molecular-genetic, physiological and behavioural data) relating to mutations in hundreds of genes associated with eye and brain development and disease.

Neuroanatomical data

Sequencing datasets

Molecular biology reagents including HCR probe sets, guide RNAs and various plasmids

Equipment design (behavioural tracking rigs and multi-spectral light sheet microscope)

Software & analysis code

These tools and resources will be made available to the community either directly or in the case of mutant and transgenic zebrafish lines by depositing these lines in international stock centres (ZIRC, EZRC). These tools and datasets will benefit both the zebrafish and wider research community, clinicians and pharmaceutical and biotech companies that use larval zebrafish for drug discovery.

Who or what will benefit from these outputs, and how?

The scientific community will benefit from the knowledge we contribute to how genetic mutations, gene interactions and the environment affect cell and circuit development in the brain and retina. Our research is interdisciplinary and will benefit several academic communities including those who are involved in fundamental, discovery research addressing, for instance, gene networks, cell-cell interactions, organogenesis, regeneration and the regulation or organ size and the development of sensory systems as well as human geneticists and clinicians studying ocular pathologies. Our research will help to bridge the gap between the highest quality research in model systems and human disease phenotypes. For instance, identification of genes that affect penetrance of eye phenotypes in fish will help to elucidate why there is variability in the predisposition to congenital eye defects in humans.

Our direct academic and clinical collaborators and wider research communities will benefit from our study of the genetic basis of eye development in normal and disease conditions. Our work also addresses fundamental cell-biological processes of tissue organisation as well as genetic and developmental compensatory mechanisms, thus, reaching communities beyond our direct field of research.

In addition to this scientific knowledge, the screening platforms, disease models (mutant and transgenic zebrafish lines), protocols, datasets and analytical tools we develop can be used by other researchers to address other aspects of brain and eye development and function. Clinicians can benefit from our rapid mutation/F0 phenotyping platform, enabling swift knock out of zebrafish orthologs of human disease genes.

Our zebrafish models of human disease genes, transgenic animals and analysis tools may be used by clinical research scientists, who will use our data to screen groups of patients for mutations and pharmaceutical and biotech companies for drug development and improved treatment and diagnosis for eye and nervous system disorders.

Furthermore, the public sector (NHS), including human geneticists, consultant ophthalmologists and genetic councillors and their patients benefit from the identification of new genetic markers to help diagnose patient cohorts. Our work is of relevance to charities working with families affected by congenital eye disorders (e.g. Fight for Sight and EURODIS). The general public benefits from our outreach into the community through web-postings, public talks, school visits and the hosting of school-age children.

The zebrafish offers a valid alternative to using rodents for preliminary behavioural analyses and to test the efficacy of novel drugs which can reduce research in mammalian species.

How will you look to maximise the outputs of this work?

Our research, including unsuccessful approaches/negative results, will be published open access in peer reviewed journals. Ongoing results from all genetic interaction analyses will be regularly updated on a dedicated web portal. We will also present our work at international conferences and through more general meetings to broader scientific audiences. We cannot anticipate all possible academic beneficiaries of our interdisciplinary work, especially those beyond our field of expertise. Thus, we will pay attention to ensuring maximum exposure of our research data through open-access publications and by sharing our findings online via our website, via social media and through exposure in the general media. In parallel, we will ensure that resources such as imaging, sequence and phenotype data, fish lines and other reagents are all openly shared with the community.

Having worked in my chosen field of research for over 30 years, we have a vast network of collaborations with individuals as well as institutes in the UK and all over the world. We often have visiting scientists working with us to learn new technologies, similarly, we send our researchers to other groups to broaden their technical skills. We will communicate/collaborate in areas in which we will benefit from input from researchers with other expertise. For instance, we communicate frequently with human geneticists who provide information on human eye disease that helps to direct our research in fish and who can reciprocally use our data to inform screens for genetic mutations in patient cohorts. We work with researchers who have expertise in analysing next generation RNA and DNA sequencing data and with colleagues who are performing related research on eye and brain development. We also have direct collaborations with clinicians working on eye diseases and metabolic diseases in children.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 222000

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 studies examine complex interactions between cells and how they become organised into tissues in the developing embryo. We focus on the interactions between structures of the sense organs and the brain, and how an animal reacts to signals from its environment. We cannot model the development of the vertebrate eye and brain adequately in non-vertebrate animals such as worms or flies or in a laboratory dish. Through our work in zebrafish we have contributed to reducing research with mammalian species.

Zebrafish is an ideal model because the way its brain and eye are built is a simplified version of that found in mammals such as humans. By studying zebrafish, we are using an animal considered less sentient than mammals to achieve our research goals and to answer our scientific questions. The larval zebrafish brain is very small and optically transparent allowing us to monitor and manipulate all neurons throughout the entire brain during behaviour. Our optical methods are minimally invasive and do not cause suffering. For most of our studies we will use very young embryos without a mature nervous system. However, to study how an animal behaves in response to its environment we will need to use larval or juvenile fish that can swim and show complex behaviours. Adult fish will almost exclusively be used for breeding to generate embryos/larvae for experiments.

Typically, what will be done to an animal used in your project?

Adult fish will be used for maintaining the fish stocks and for breeding to provide us with eggs to use in our research.

Larval fish will typically be genetically modified to have one or more mutated gene and for use in behavioural experiments or for the study of neuroanatomy and may also have transgenes that cause cells in their brain and/or eyes to

emit light when those cells are active and/or

allow their activity can be controlled using light or chemical stimulation and/or

fluoresce so that key structures/circuits in the brain or specific cell types can be visualised.

to be eliminated using chemogenetic or optogenetic tools

Larval fish will be used for studying animal behaviour where we watch their response to various stimuli first while swimming freely and/or while immobilised in agarose when we can simultaneously record neural activity. In some studies, we might add substances to the water to study the effect of a drug on the animal's behaviour, or we might look for a drug that will treat a disease or abnormal behaviour.

Specific neurons that are shown by our experiments to be involved in behavioural responses may be ablated using either a laser or chemical methods or labelled using genetically encoded photoactivatable proteins or substances injected into the brain or eye of anesthetised fish to show neuronal morphology and label neural circuits.

Most experiments will take less than 24hrs.

What are the expected impacts and/or adverse effects for the animals during your project?

Most animals will not have any adverse effects. Most animals are used for breeding and in behaviour analysis, and, generally, the animals should not experience pain. Experimental animals might experience temporarily mild discomfort or mild pain. On rare occasions, animals might experience moderate discomfort or longer-lasting mild discomfort or mild pain. The duration of any pain or discomfort might last for the duration of the experiment which is typically around 6hrs.

We do not expect weight loss, tumours or other gross abnormalities. The possible exception are animals which might, for example, experience some effect on their movement (swimming) behaviour in our models of human diseases that affect the nervous system.

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 Adult GA zebrafish used for breeding and maintenance: 89% sub-threshold, 10% mild, 1% moderate.

For larvae used in experiments: 95% mild and 5% 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?

The eye of a vertebrate such as a fish or human, the complexity of the brain organisation, and animal behaviour, are very different than the eye, brain and behaviour we see in less sentient animals such as worms or flies. We are also trying to model aspects of human diseases of the eye and nervous system which we cannot study in the same way in less complex animals, or in a tissue culture dish or using computer simulation.

Which non-animal alternatives did you consider for use in this project?

We use bioinformatics and computational analysis to analyse our data from animal research in the most efficient way.

We are using animal research data to build our own standardised reference brain atlas and we also make use of existing community brain atlases (e.g. mapzebrain, ZBB) to evaluate neuroanatomy and gene expression patterns. Registering our data onto reference brains means we can get a lot of information about specific cells and brain regions without the need to duplicate experiments in our own lab.

Primary embryonic pluripotent cell aggregates “organoids” that can assemble into anterior neural structures have recently been developed in zebrafish. This *ex-vivo* system has been used to study eye development and provides a unique environment to study development and the roots of pathologies under tightly controlled conditions. These micro-environments although invaluable to study certain questions about forebrain development are artificial and ultimately any findings would need to be replicated in the whole animal. We will continue to keep abreast of this system and integrate it into our studies where possible. Similarly, we hope to set up primary zebrafish cell culture and we hope that this system will prove useful for studying some cellular and sub-cellular mechanisms. Again, this would not be able to replace studies using the intact animal but could help reduce numbers used.

Why were they not suitable?

The vertebrate eye is a very complex structure. We cannot study the role that particular proteins play in the development of a normal eye, or what happens when they do not function properly except in the context of the whole animal. Similarly, we are studying how the left and right side of the brain become different in their structure and function during development. This cannot be modelled adequately on a computer or using *in vitro* models. Lastly, we study the complex interaction of an animal with its environment, again this must be done using whole animals and modelling can only give us suggestions of what to explore further.

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 protocols for zebrafish has been well-established for more than 30 years. I have estimated the number of animals that will need to be bred to adulthood based on the number of distinct genetically modified lines that are needed for this project, the breeding cycle that is required to maintain healthy fish, and the experience of my lab over the last

30 years. In addition to the transgene and/or mutation-carrying lines that we routinely maintain for our research our combined eye disease and asymmetry genetic screens, we expect to generate approximately 50 new mutant/transgenic lines per year. This number is derived from the number of genes we will target per year and our previous experience of the viable hit rate for our F0 knock-out method. New stable mutant lines will be maintained in multiple transgenic and mutant backgrounds to explore compensatory effects of related genes. Thus, we estimate that, on average we will need to maintain up to 400 genetically distinct lines for 5 generations with a typical group size of 50 fish necessitating about 100,000 adults in total for breeding and maintaining all lines over the next 5 years. We will regularly review our lines and sperm freeze any lines not in regular use to reduce the number of adult fish we maintain whenever possible. We estimate as part of our F0 screen that we may occasionally need to raise and maintain adult fish that display mild or moderate phenotypes to breeding age we have estimated 1000 fish for this protocol. To sperm freeze lines of interest and reduce the number of live fish we maintain we will require 1000 fish.

Almost all experimental procedures will use zebrafish at larval/juvenile stages (5 to 21 days old). We examine behaviour in freely swimming animals in response to a variety of naturalistic stimuli to discover rules by which specific patterns of sensory information are converted by the brain to behaviour. We also use drugs and advanced optical and genetic means to manipulate brain circuits in intact animals to observe how behaviour changes. To obtain statistically robust data we need a group size of around 40 animals per experimental condition and this equates to an estimated 30,000 larvae in total over the course of 5 years.

In addition, we do some experiments using tethered larvae where we can both monitor their behaviour as well as using microscopes to observe, and manipulate, brain activity at very high resolution. To test hypotheses about the roles of specific brain cells, we will need to ablate these cells using either a laser or genetic method and then observe how brain activity and behaviour change. These experiments will reveal the structure and operation of the brain circuits that control behaviour and will require 30,000 larvae in total over 5 years.

Neuroanatomical, Histological and genetic analyses form a large part of our work for our genetic screens and other phenotypic analyses and RNAseq studies we estimate we will need 60,000 in total over 5 years based on number of animals per experimental condition/genotype required to obtain statistically robust data.

Thus, in total we will require an estimated 222,000 animals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

When appropriate, we use statistical design to determine the number of animals we need to use for each experiment to get a valid result. It is important to balance the need to have statistically significant results with the desire to reduce the number of animals we use as much as possible.

We are also building a standardised reference brain and eye which will reduce the number of animals that need to be used as these resources facilitate comparison of data from multiple experiments.

Our experimental design maximises data collected from individual larvae. The datasets we collect are often comprehensive and multimodal. Imaging the whole brain means we can

collect functional data from thousands of neurons during a single procedure so we don't need multiple imaging sessions to survey all the different brain regions involved in driving a particular behaviour.

Without whole brain imaging we may not be able to cover all brain regions in a single animal which would mean the animal would need to be repeatedly exposed to the same stimulus to cover all brain regions. As we cannot expose the animal to many repeated aversive stimuli, this would mean using more animals to acquire the same amount of data (i.e. different brain regions in different animals). Therefore, whole brain imaging reduces animal numbers while preventing over exposure of aversive stimuli to each individual animal. So, by utilising whole brain imaging we both refine our experimental procedure avoiding the need for multiple imaging sessions for the indvidual animal and through maximising the amount of data we can collect from a single animal/trial we also reduce the number of animals we need to use overall for each experiment.

Our knockout screens for genes affecting eye and brain development use a very efficient F0 Crispr method which means we can screen for interesting phenotypes without the need to raise large numbers of fish carrying mutations to adulthood. For both our eye and brain asymmetry screens we can, for the most part, screen our mutants before 5 days post fertilisation when they are not yet protected. The efficiency of the Crispr method for making mutant zebrafish means when we do need to make stable lines, we do not have to raise large numbers of fish to obtain a sufficient number of carriers. This targeted mutation approach means we no longer need to generate a large number of randomly mutagenised fish to be screened for mutations of interest. This refinement to our screening methodology results in a substantial reduction in the number of animals that are used to generate useful genetic mutations.

For larval behavioural experiments, we use pilot studies and power calculations to determine the minimum number of fish needed for our analysis to be statistically robust and only raise the number we need for each experiment to free-feeding larval stages.

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 fish facility has developed procedures that enable us to maintain colonies with excellent standards of welfare and the smallest possible numbers of breeding adults. We also cryopreserve lines whenever possible to reduce the number of live fish. Clutches of eggs are usually much larger than required for experimental testing. To reduce the number of protected animals used, we will only grow to free-feeding larval stages the number of fish required for experimentation or line maintenance.

Pilot studies: For small molecule testing where limited dosing data exists in zebrafish, we will perform pilot studies on small numbers of larvae less than 5 days post fertilisation to optimise drug doses. For new behavioural assays or experiments in new genetic backgrounds, small pilot trials will be used to determine effect sizes or gauge unexpected adverse effects so we can deduce the minimum number of fish that need to be raised to free-feeding larval stages for our experiments.

Computational modelling: We use computational modelling and advanced statistical approaches that enable us to identify principles of circuit function using complex, high-dimensional data from fewer 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 are using the zebrafish to investigate the development of the eye and the brain in health and in disease. Unlike mammals, zebrafish lay their eggs externally and we can simply collect them from their breeding or home tank, with no harm done to the females laying the eggs. The embryos are transparent which allows us to gain much information by simply examining them under a microscope.

Larval zebrafish are very small and transparent this allows us to monitor and manipulate the activity of entire brain circuits, at cellular resolution, while tracking behaviour. This is currently not practical in other widely used vertebrate model organisms. These types of optical methods are minimally invasive and are associated with less pain or discomfort than invasive electrophysiological methods or equivalent optical approaches in rodent models (involving implanted windows/lenses/fibres).

For some behavioural tracking, the larval zebrafish must be partially restrained to allow us to examine the brain under the microscope. To minimise stress, we have refined this procedure by briefly anaesthetising the animal while they are mounted in the agarose. Agarose gel causes no damage or lasting harm to the fish. Once the fish have recovered from the anaesthetic, they start to display naturalistic behaviours (such as hunting behaviours) while tethered in the agarose and will continue to do so for at least 72 h. Once the larvae are released, they can be grown to maturity and show no obvious physical or behavioural defects.

In a small number of larvae, we will use a laser or other approaches to selectively target and ablate small groups of neurons. The laser ablation procedure is carried out in anaesthetised animals. This very precise method of ablation is a substantial refinement upon invasive mechanical ablations. Following ablation when the larvae recover from the anaesthetic, we typically observe very specific behavioural changes while most of the larval behavioural repertoire is unaltered. This suggests the larvae suffer little if any discomfort or distress and confirms the specificity of the ablation.

Why can’t you use animals that are less sentient?

A large amount of our work is using animals at the immature, embryonic stage or after they were killed humanely. However, for some of our studies we do need to use larvae e.g. in our study of animal behaviour as we require the animals to show a robust visually or other sensory driven behaviours. We are using the least sentient model animal that shows a similar eye and brain structure and organisation as we see in humans. We want to understand the principles of the vertebrate brain so we cannot use simpler laboratory model organisms such worms or flies because these are not vertebrates.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

During our behavioural tracking experiments, animals are continuously tracked with a video camera on computers that we can monitor for adverse outcomes. Experimenters are trained to spot signs of distress or health issues. Any behavioural indications of adverse responses are therefore readily detectable, allowing for immediate intervention or termination of the experiment.

For breeding of genetically modified animals, if a new reliable method to identify transgenic and mutant animals in embryos or fry is established that avoids needing fish to be grown to maturity, e.g. by removing small number of cells in early embryos to be used for genotyping instead of fin biopsies, we will adopt such methods.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Local AWERB guidelines; ARRIVE guidelines of the NC3Rs; guidance on Animal Testing and Research from the Home Office; Good research practice guidelines from the Wellcome Trust; LASA and RSPCA guidelines; PREPARE guidelines and scientific journals (Aleström et al., 2020).

Aleström P et al. Zebrafish: Housing and husbandry recommendations. Lab Anim. 2020 Jun;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?

Members of our zebrafish community attend conferences with a 3Rs focus and we consult resources including Norecopa (https://norecopa.no/), the ZFIN zebrafish protocols database(https://zfin.atlassian.net/wiki/spaces/prot/overview) and publications related to fish welfare(https://mdpires.com/bookfiles/book/1802/Welfare_of_Cultured_and_Experimental_Fishes.pdf).

Our Aquatics AWERB and zebrafish user group are excellent forums for sharing best practices as well as new 3Rs advances and our NACWO’s provide practical guidance on how best to implement refinements.

77. Sea lice infection and immunity and development of vaccines

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 animalsreared 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

sea lice, salmonids, infection, immune, vaccine

Animal types	Life stages
Salmon (Salmo salar)	Juvenile, Adult
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 aim of the project is to develop vaccines for the sustainable management of sea lice based on improved scientific understanding of sea lice infection and the associated immune response of salmonids.

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?

Sea lice infection affects the welfare of farmed fish and the ability of aquaculture to contribute to global food production. Treatments such as vaccines are required to reduce the harm caused by lice infection, but they must be shown to be safe and effective first by testing on infected fish. Vaccination is particularly desirable as an 'environmentally sound' contribution to the integrated pest management of sea lice. We will enhance biological understanding of sea lice disease by assessing infection and immune response, and subsequently develop new prophylactic methods of protection for prevention and control of sea lice outbreaks. The work will include developing, testing and monitoring the effectiveness of vaccines as sustainable alternatives to pesticides and drugs.

What outputs do you think you will see at the end of this project?

Short-term outputs

Studies conducted under this licence will provide data and reports on the safety and efficacy of candidate sea louse antigens. Research will also provide reliable evidence as to whether potential new vaccines can: reduce sea lice abundance; reduce sea lice reproductive capacity; prevent or improve disease signs; be applied safely and effectively through different delivery routes.

Where the work is part of a collaboration with academic staff, data on salmonid immune response and sea lice infection will be published via reports, conference presentations, on-line press releases and scientific journals.

Reports produced on safety/efficacy data will be publicly available through an on-line research repository system.

Longer term outputs

Reports may be submitted to regulatory authorities as evidence for licensing of promising vaccines as potentially new medicines.

Even where data on vaccines or other treatments is initially confidential, results will be included in peer reviewed publications following approval by all involved partners.

Data generating knowledge on immune response through novel biotechnological methodologies will be kept confidential where the work forms part of industrial collaboration until intellectual property has been protected, i.e. through patents.

In order to limit repeat studies on unsuccessful vaccine targets negative data will be communicated through scientific reports and conference presentations.

Material generated in studies will also be used to educate school children on parasitic diseases of fish, e.g. through school placements and University open days.

Material and data will be generated for teaching undergraduate and postgraduate students and CPD courses held for fish farmers, fish health professionals and other stakeholders.

Who or what will benefit from these outputs, and how?

Short term benefits

With regards to direct benefits for aquaculture and animal welfare, the economic cost of sea lice has previously been reported as much as 6% of production value due to losses, mortalities, and reduced growth (Costello, 2009), which is now likely to have increased. The global economic impact of sea lice on the Atlantic salmon industry is currently

estimated to be >$1 US billion (Brooker et al., 2018). The industry uses various methods to control salmon lice infestations, including chemotherapeutants, mechanical (e.g. thermo or hydro-licers), physical barriers (e.g. snorkel cages) and biological controls (e.g. cleaner fish). However, major challenges are associated with the application and efficacy of these approaches and environmental concerns including chemical waste and residues. Increasing resistance of lice to drug treatments, reduced effectiveness of hydrogen peroxide treatments, and increased tolerance of lice to freshwater treatment, have all been reported, while cleaner fish are also susceptible to disease. Additionally, there have been increased reports of fish losses and bacterial infections due to non-medicinal/mechanical sea lice treatments (Norwegian Veterinary Institute, 2023). Thus efficacious sea lice vaccines would reduce the risk of alternative treatments and promote overall health and welfare of animals over the production period. The salmon farming industry continues to look for new 'environmentally friendly' solutions to control salmon lice infections, which offer better fish welfare, less fish handling, less stress, and less physical trauma than the methods currently employed. Thus, a commercial salmon louse vaccine would provide a practical, safe, and eco-friendly approach to managing salmon lice and enhance current salmon lice control strategies.

Farm companies and the farmed fish will benefit from improved resistance to disease as well as from reduced resistance of lice to chemotherapeutants with reduced treatment applications.

Mid-term benefits

Studies performed under this project licence will facilitate improved welfare of farmed UK and global salmonid production, increase salmonid aquaculture sustainability and significantly reduce economic costs associated with current sea lice treatment practices. Furthermore, the outcomes of the project provides potential benefits for the marine environment by reducing transmission of lice from farmed to wild fish populations, and the application of chemical treatments.

Long term benefits

Improving sustainable protein production for the growing population will also benefit humans through enhancing food security. Scientifically, the work performed under the licence will be of direct benefit for Atlantic salmon and trout producers, pharmaceutical industries, academic researchers (immunology and parasitology) and biologists.

Results will be published in scientific journals and presented at conferences. The research will be communicated via press releases, tradeshows and relevant magazines.

Sustainable, environmentally-sound treatments such as vaccines will help farmers meet the Government calls for reduced environmental impact of marine cage farming including impacts on prawn and lobster fisheries associated with other anti-louse parasiticides.

In the long term, as new control methods come to market and the disease burden is reduced, improved fish health will facilitate increased production for public consumption, providing valuable protein and beneficial omega-3 oils that are good for human heart health.

More effective treatments may have environmental or ecological benefits such as reduction in off-target effects of pesticide or sound pollution.

Development of a sea lice vaccine over a 5-10 year period will increase UK aquaculture's economic contribution and competitiveness in terms of global salmon production, will

safeguard fish health and welfare and help protect rural jobs and the marine environment. A conservative estimate of 50% reduction in control costs due to vaccination would improve UK industry returns by £16M p.a. (less vaccine costs) excluding productivity gains. Further benefits would accrue from global vaccine sales which, with a global Atlantic salmon production estimated at 2.3M t in 2015 and an estimated mean harvest size of ~5.1kg gives a minimum number of vaccinated salmon ~450M which, if similarly vaccinated at a level of 5 pence per fish would provide a revenue of ~£22M p.a. less costs.

References

Brooker A.J., Skern-Mauritzen R., Bron J.E. (2018). Production, mortality, and infectivity of planktonic larval sea lice, *Lepeophtheirus salmonis* (Krøyer, 1837): current knowledge and implications for epidemiological modelling. *ICES Journal of Marine Science* **75(**4), 1214–1234.

https://doi.org/10.1093/icesjms/fsy015

Costello M.J (2009). The global economic cost of sea lice to the salmonid farming industry. *Journal of Fish Diseases* **32**(1), 115-118. DOI: 10.1111/j.1365-2761.2008.01011.x

How will you look to maximise the outputs of this work?

Working with pharmaceutical industry partners as we do currently, provides the experience and resources for moving any developed vaccines that prove efficacious from R&D to licensing, clinical phase testing, product marketing and distribution.

We will work with established commercial partners on vaccine design, biotechnological upscaled production and efficacy assessment. Furthermore, we will look to latest developments in efficient recombinant protein expression, reverse vaccinology and AI to identify and establish the most promising vaccine candidates *in silico*, prior to *in vivo* testing to maximise the chance of observing a protective effect from *in vivo* studies. We will utilise on-line search tools weekly (e.g. PubMed -National library of medicine; Google Scholar) to ensure we are up-to date with on-going research and literature that may expedite antigen discovery, improve vaccine biotechnology, and provide non-animal alternatives for sea lice vaccine development and research.

We will collaborate with clients and academic partners on papers, and conferences including a focus on good practice and identifying what hasn’t worked to help eliminate unsuccessful approaches.

We will publish any refinements we make to our methods to improve fish welfare, especially when improving vaccine antigen discovery, trialling new vaccination approaches or vaccine challenge trial design.

Species and numbers of animals expected to be used

- Salmon (Salmo salar): 32,500
- Rainbow Trout (Oncorhynchus mykiss): 8000

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.

Atlantic salmon is the most commercially important salmon species susceptible to sea lice, thus necessitating the need for it as a model (Boxaspen, 2006). Rainbow trout are additionally a useful model representing a member of the Oncorhynchus genus, which incorporates a range of species with diverse susceptibility to lice infection (Braden, Monaghan and Fast, 2020). The purpose of this licence is to develop prophylactic treatments against sea lice which cause a major parasitic disease of Atlantic salmon and rainbow trout farmed at sea in the UK. This licence will therefore use two farmed fish species, Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). Depending on what stage of their life cycle testing is needed, salmon could be reared in either fresh or marine water.

However, sea lice infections are limited to marine water salmon, but vaccination/immunity studies can also be undertaken in freshwater fish. Rainbow trout will be used mainly as a comparative species to enhance understanding of the disease biology and host response. The total number of fish expected to be used is 40,500 over a study period of 5 years.

Typical safety testing of fish will require 1,000 Atlantic salmon per year (5,000 total) and 250 rainbow trout per year (1,250 total).

Typical trialling of vaccines will require 4,100 Atlantic salmon per year (20,500 total) and 1000 rainbow trout (5,000 total).

Typical infection/immunity trials will require 1,400 Atlantic salmon per year (7,000 total) and 350 rainbow trout (1,750 total).

Total required fish numbers are 32,500 Atlantic salmon and 8,000 rainbow trout.

References:

Braden L.M., Monaghan S.J., Fast M.D. 2020. Salmon immunological defence and interplay with the modulatory capabilities of its ectoparasite *Lepeophtheirus salmonis*. *Parasite Immunology*, **42**(8), e12731. https://doi.org/10.1111/pim.12731

Boxaspen K. (2006). A review of the biology and genetics of sea lice. *ICES Journal of Marine Science,* **63** (7), 1304–1316. https://doi.org/10.1016/j.icesjms.2006.04.017

Typically, what will be done to an animal used in your project?

Research on new treatments conducted under this project licence will include immunisation and infection studies with sea lice. Procedures used for the purpose of investigating immune responsiveness or vaccination administration are classified predominantly as ‘mild’. Treatments may be administered via the water to be absorbed by the fish (immersion or spray), included in the feed (oral) or by gavage or injected (typically 0.05-0.2 mL doses) under anaesthetic. Typically animals may be anaesthetised to allow measurement or marking (panjet, Visible Implant Elastomers (VIE) or Passive Integrated Transponder (PIT) tags) and then allowed to recover. Treatment trials will generally involve infection of fish previously treated with either a potential or current vaccine or sham vaccine formulation, allow disease progression to be compared between treatment groups, and then culling of fish after a few weeks.

Fish may experience a short period of reduced appetite (i.e. 1-2 days). The order and timing of procedures may vary; for example vaccines are given months to work before infection. Fish will usually only be infected and treated once but may require repeated anaesthesia if booster doses are required. Safety tests of 2 weeks will be performed for new experimental vaccines and formulations to ensure no toxicity before being applied to

larger scale trials of 2-4 months. Fish will be regularly monitored on a daily basis and observations will be scored during monitoring including feeding, behaviour, respiration and appearance. Action will be taken on trials immediately if severity threshold appears likely to be breached.

Typical vaccination regime:

Fish movement to tanks and acclimatisation

Anaesthesia - VIE tag, needleless injection (e.g. panjet) or PIT tag marking - recovery

Anaesthesia - vaccination (oral, immersion, gavage, injection, spray) - recovery

Anaesthesia - boost vaccination (oral, immersion, gavage, injection, spray) - recovery5. Sea lice challenge

6. Euthanasia of fish for vaccine efficacy assessment and tissue sampling - Schedule 1 killing

Typically there will be at least 1 week between anaesthetic applications (AB), but typically >4 weeks between prime and boost vaccinations.

Where infection trials are undertaken for the provision of sea lice material for antigen discovery and determining key aspects of host-pathogen interaction, low doses of sea lice (challenge of 20 copepodids per fish maximum; 80% lower than typical acute challenge model) may be used to challenge the fish over longer periods of time (up to 3 months).

Typical experimental infection regime:

Fish movement to tanks and acclimatisation

Anaesthesia - VIE tag, needleless injection (e.g. panjet) or PIT tag marking - recovery3. Sea lice challenge

4. Euthanasia of fish for host-pathogen analysis and collection of parasite material - Schedule 1 killing

Only experienced and trained staff will handle fish e.g. for weight measurements or carrying out procedures such as vaccination, ensuring that any distress or harm is minimised by use of anaesthetics when appropriate and careful netting, i.e. to minimise scale loss, is undertaken.

What are the expected impacts and/or adverse effects for the animals during your project?

Some injection vaccines may incur minor side-effects such as localised lesions, which are commonly observed following IP vaccination. This is considered to be mild severity and will be monitored from initial Safety tests to ensure vaccines causing lesions of moderate severity are avoided (i.e. >3 according to Spielberg scoring) are not trialled further.

Sea lice challenges may result in minor skin irritation and erratic behaviour including jumping upon first exposure, with development of sea lice to adult stages causing mild skin damage. Fish will be culled where progression of disease appears likely to advance to moderate severity where lice accumulate behind the dorsal and adipose fins. Typically 10% of infected fish will suffer moderate harm, 90% will suffer mild harm. All fish will be culled at termination of experiments. The majority of protocols are routine procedures that

will have ‘mild’ outcomes. Fish will not be re-used for further trials. Similar to vaccine trials, during this time fish will be regularly monitored on a daily basis and observations will be scored during monitoring including feeding, behaviour, respiration and appearance. Action will be taken on trials immediately if severity threshold appears likely to be breached.

PIT-tagged fish may experience short term transient discomfort and local inflammation.

Administration of substances by immersion, gavage, injection or spray (Saito et al., 2024) may cause short term transient discomfort. Gavage can cause irritation of the oesophagus following administration. Injection can cause inflammation, melanisation and internal adhesions associated with localised reactions at the injection site and to immune-stimulating adjuvants. When using some mineral oil-based adjuvants, more chronic adhesions can occur in the peritoneal cavity following intraperitoneal vaccination.

In the case of clinical signs associated with vaccine toxicity, fish may exhibit erratic or uncoordinated swimming, lack of feed response, darkening or ulceration of the skin, eye damage or fin erosion.

References:

Saito H., Minami S., Yuguchi M., Shitara A., Kondo H., Kato G. and Sano M. (2024). Efficient showering vaccination with a live attenuated vaccine against herpesviral hematopoietic necrosis in goldfish. *Aquaculture* **578**, 740140. https://doi.org/10.1016/j.aquaculture.2023.740140

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)?

Infected fish (Atlantic salmon and rainbow trout) will be carefully monitored for signs of disease and we will aim to react as quickly as possible if any develop from mild to moderate disease. We anticipate from over 10 years experience of running previous sea lice challenges within our aquarium facility that 90% of animals infected will suffer mild harm and 10% will suffer moderate harm.

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?

Host-parasite interactions are complex and not completely understood. The infections we are undertaking for testing of treatments depends on the health, physiology and immunity of the fish, and on the genetics of both fish and parasite. These interactions can not be recreated as computer models or in the laboratory.

The sea louse lifecycle can not currently be completed without the use of live fish, although a number of groups worldwide, including our own, are working to achieve this.

There are currently no non-animal alternative models to study sea lice vaccine efficacy or fish mucosal immunity. In vitro tissue culture systems exist for Atlantic salmon, but cannot support sea lice attachment. Efficacy is typically measured by counting the number of attached parasites on infected fish.

Counting parasites on fish as a form of efficacy testing is limited to showing lethal effects of vaccine response in the parasite, which still requires fish infection. Only the animal model can enable investigation of broader biological and immunological effects simultaneously, especially with regards to mucosal immunity (antibody production in mucus), or adaptive immunity (T cells and B cells).

Measurement of immune response from tissues, mucus and blood, e.g. gene expression analysis and antibody measurement in pilot vaccination studies, will reduce the number of animals subjected to infection studies, but requires initial infection of animals.

Skin culture models are being developed by our group, but still require donor animals. Considering that a single fish can provide enough material for considerable replicate skin tissue/cell assays (Karlsen et al., 2021), a significant reduction in animal use is envisaged where their utility is applicable. These models will be applied for antigen screening and reduce and replace the number of animals needed for larger scale treatment studies.

While most of the work requires animal-based experimentation, we will promote the 3R's where possible by pursuing the use of *ex vivo*, *in vitro* and *in silico* models and examining their relevance against the *in vivo* experimentation conducted as part of this project licence.

**ex vivo* = studies using animal tissue post-mortem

**in vitro* = studies using artificial tissue or non-animal approaches

**in silico* = experiments conducted through computer simulation/prediction software

Reference:

Karlsen C., Bogevik A.S., Krasnov A., Ytteborg E. (2021). In vivo and in vitro assessment of Atlantic salmon skin exposed to hydrogen peroxide. *Aquaculture*,

540736660,https://doi.org/10.1016/j.aquaculture.2021.736660.

Which non-animal alternatives did you consider for use in this project?

Blood feeding assays to evaluate vaccine effects to multicellular parasites have been developed for ticks and mites, but have thus far been impossible to apply for marine parasites due to the aquatic environmental conditions required by the parasite. Similar *in vitro* methods are being developed by our group for adult lice, where possible, to feed blood antibodies to sea lice and assess vaccine efficacy.

The requirement for extensive sea lice material for exploration of vaccine candidates will be obtained from naturally occurring infections at field sites (fish farms) when possible, thus minimising the need for additional experimental infections. *In silico* prediction software and AI may also be applied to

streamline vaccine candidate identification, further replacing the use of animals. We will utilise on-line search tools weekly (e.g. PubMed -National library of medicine; Google Scholar) to ensure we are upto date with on-going research and literature that may

expedite antigen discovery, improve vaccine biotechnology, and provide non-animal alternatives for sea lice vaccine development and research (e.g. organoids).

Where fish skin tissue culture assays are applied, skin extracts will be taken from few fish post-mortem to provide tissue culture material with greater replication (e.g. Karlsen et al., 2021).

Finally, vaccine efficacy assessment of vaccines following challenge will not be limited to counting the number of lice and comparing between vaccinated and control fish. Any potential effect on the parasite reproductive output (eggs/hatch success) or offspring will also be assessed by removal of lice from challenged fish and assessing the eggs or offspring *in vitro*.

Reference:

Karlsen C., Bogevik A.S., Krasnov A., Ytteborg E. (2021). In vivo and in vitro assessment of Atlantic salmon skin exposed to hydrogen peroxide. *Aquaculture*,

540736660,https://doi.org/10.1016/j.aquaculture.2021.736660.

Why were they not suitable?

The sea lice life cycle cannot be maintained without a fish host. We can identify potential vaccine candidates and treatments without using fish, but they must be proven effective and safe using infected fish before they can be approved for use on farms.

The *in vitro* methods highlighted above are not yet well established and are limited to showing lethal effects in the parasite. Only the animal model can enable investigation of broader biological and immunological effects simultaneously, especially with regards to mucosal immunity (antibody production in mucus) or adaptive immunity (T cells and B cells). Measurement of immune response from tissues, mucus and blood, e.g. gene expression analysis and antibody measurement in pilot vaccination studies, will reduce the number of animals subjected to infection 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?

Based on previously published work and past experience of vaccine-challenge studies, we will need these numbers over 5 years if demand for trials proves consistently high, but the eventual total is likely to be lower in reality.

For Atlantic salmon, we anticipate using up to 6,500 fish per year. We anticipate safety and vaccination studies on 1-10 candidate vaccines per year, requiring up to 1,000 fish. This estimate is based on using approximately 100 animals for a basic safety test (e.g. 20 fish per control, treatment antigen, adjuvant and delivery route), of which there may be up to 10 novel treatments/approaches per year. We expect to use 800 fish per year to produce lice for *in vitro* screening assessments, provision of lice material (e.g. parasite protein and

RNA) and immunity trials, a further 600 per year to produce lice to initiate in vivo infection experiments, and 4,100 per year for *in vivo* efficacy testing for candidate vaccines.

Allocation of fish to treatment groups and inclusion of trial controls is critical for vaccine-challenge studies for which we will conduct up to 2 per year. These studies will typically include up to 4 vaccine candidates and a control assessed as an independent tank challenge model. For each vaccinechallenge trial using this model 6 tanks of up to 30 fish per treatment have been estimated including controls (6 x 30 = 180). For 4 candidate vaccines + control = 180 x 5 = 900 fish per trial x 2 = 1800 fish per year. For sea lice production to challenge these fish will require 360 fish per trial, thus 720 fish per year. Therefore an estimate for 5 years with independent vaccine-challenge tank studies requires 12,600 fish in total. Where a mixed tank challenge model is applied mixed groups of 15 fish for up to 10 treatments including the control group will be required. Therefore up to 150 fish per tank will be used in triplicate tanks, thus 450 fish per trial. Therefore for 2 trials per year including fish for sea lice production (2 x 340 fish) we require 1,580 fish per year (7,900 total). We anticipate a greater number of trials with Atlantic salmon than rainbow trout, hence the different estimated fish numbers required.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have analysed existing data to ensure that our experimental designs are based on robust and reliable information about the natural variation between fish and between tanks, thus reducing the need to repeat studies. We are currently employing the NC3R's EDA tool to help design experiments with sufficient power.

The US and European medicine regulatory authorities (FDA and EMA) set out requirements for the type, size and duration of studies required for approval of veterinary medicines for farm animals. This means following their advice avoids using too many animals by adding extra studies or testing at unnecessary doses.

Skin culture models are being developed by our group, and considering that a single fish can provide enough material for appropriate replication in skin tissue/cell assays, a significant reduction in animal use is envisaged where their utility is applicable. These models will be applied for antigen screening and reduce and replace the number of animals needed for larger scale treatment studies.

While most of the work requires animal-based experimentation, we will promote the 3R’s where possible and reduce the number of animals needed in studies by pursuing the use of *ex vivo*, *in vitro* and *in silico* models and subsequently examine their relevance against the *in vivo* experimentation conducted as part of this project licence.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In order to obtain sufficient lice numbers for vaccine-challenge testing requires a pre-challenge of fish with lice to generate the next generation of parasites (i.e. amplification step), which is reflected in the estimated numbers of animals required. We only grow up strains (amplify production from culture stock) of sea lice that are required for research, and keep colony size to a minimal viable level until required just before a trial. The strains and numbers used in trials will be periodically reviewed and potentially reduced where possible and published literature will be reviewed as the project advances to stay informed of current best practices.

We aim to eliminate unsuccessful candidate vaccines before testing in live fish, for example by use of *in silico* vaccine prediction software and AI tools. Regarding lice amplification for *in vivo* challenge studies, it takes fewer fish to grow enough lice to test vaccine efficacy once lice are removed from fish and parasite eggs hatched off the host. *In vitro* assessment of blood and mucus samples to look for antibody responses will inform of potentially promising candidates reducing the need for excessive animal studies.

We will supply excess material from sea lice challenges (e.g. preserved tissues, blood and sea lice) to other researchers to reduce the need for dedicated infections for producing that material.

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.

Short fasting intervals of 24 hours will be applied prior to fish receiving anaesthesia to reduce stress during vaccination treatments. Husbandry and rearing conditions will satisfy the species requirements to ensure optimal welfare of the fish. This will be achieved by automated daily monitoring, e.g.

appropriate regular feeding manually or by using automated feeders; water quality measurement – salinity, nitrate, nitrite; measuring environmental conditions – temperature and oxygen. Fish will be infected with sea lice under controlled conditions for assessments of immune response and hostpathogen interactions and to determine vaccine efficacy. We restrict how many sea lice infect a tank of fish, and use fewer lice to infect smaller hosts. We aim to keep infection at levels that are easily supported by the fish without causing serious harm or distress.

Administration of vaccine/biologicals via gavage and injection (e.g. intraperitoneal, intramuscular) can result in minor inflammation and small wounds at the point of vaccination. For gavage, smooth ended delivery tubes and appropriately sized smooth capsules will be used to minimise discomfort. For injection, appropriately sized needles will be determined for the respective size of fish prior to experimental use.

Prevention of unnecessary suffering or breaching of severity levels will be achieved by regular monitoring and scoring of behaviour, appearance, feeding and respiration of fish each day by the Named Animal Care and Welfare Officer (NACWO) with immediate intervention undertaken where necessary.

Why can’t you use animals that are less sentient?

Sea lice are obligate marine parasites that can only be grown on a few species of fish with Atlantic salmon and rainbow trout representing two of the main susceptible species. Both infections develop over weeks, meaning that we must use fish that are conscious to maintain normal swimming and feeding, and we can only use fish that are old enough to go to sea.

The combination of procedures such as sea lice infection, handling, anaesthesia, immersion and oral vaccination/gavage, and injection inoculation may result in sub-threshold to ‘moderate’ severity of outcome for the fish. However, immersion and oral vaccination, as opposed to injection, will be applied where possible to investigate mucosal immunity minimising handling stress. Additionally, for sea lice infection experiments, chronic exposure to damaging adult sea lice infections may be avoided by assessing the effect of the host immune response to the adult parasite under non-animal lab-based (*in vitro)* approaches rather than using animal infection (*in vivo)* where possible, preventing possible ‘moderate’ outcome for these fish. All research undertaken under this project licence will adhere to the ARRIVE guidelines.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will use the NC3Rs online Experimental Design Assistant tool to ensure that we design valid experiments, use sensible numbers of animals, and analyse our data correctly.

We limit the parasite burden per animal to levels that are expected to cause mild harm based on the size of the hosts. A minimum number of animals will be used as required for the provision and maintenance of sea lice required for research studies.

All animals will be acclimatised to experimental set-ups prior to commencement of experiments. Where possible experimental tanks will be subjected to the same conditions (temperature, salinity, dissolved Oxygen, pH) as holding tanks to avoid inducing unnecessary stress.

Samples, e.g. blood for antibody testing, and tissues for qPCR will be taken post-mortem to reduce handling under anaesthesia.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the following resource libraries of published best practice guidance to ensure experiments are conducted in the most refined way:

NC3Rs (https://www.nc3rs.org.uk/3rs-resources)

Norecopa (https://norecopa.no/)

European Union (https://etplas.eu/education/).

In addition, we consult the following guidelines:

Veterinary Medicines Directorate - https://www.gov.uk/government/collections/veterinary-medicinesguidance-notes-vmgns

European Medicines Agency - https://www.ema.europa.eu/en/veterinary-regulatory-overview/researchdevelopment-veterinary-medicines

Food and Drug Administration - https://www.fda.gov/animal-veterinary/guidance-regulations

NC3Rs ARRIVE Guidelines 2.0: (https://arriveguidelines.org/) The ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) are a checklist of recommendations to improve the reporting of research involving animals – maximising the quality and

reliability of published research, and enabling others to better scrutinise, evaluate and reproduce it.

Norecopa PREPARE Guidelines: (https://norecopa.no/prepare)

We also consult fish specific resources: Noble, C., Gismervik, K., Iversen, M. H., Kolarevic, J., Nilsson, J., Stien, L. H.& Turnbull, J. F. (Eds.) (2018). Welfare Indicators for farmed Atlantic salmon: tools for assessing fish welfare 351pp.

RSPCA Welfare Standards for Farmed Atlantic Salmon

C. Sommerville, R. Endris, T. A. Bell, K. Ogawad, K. Buchmann, D. Sweeney. Veterinary Parasitology 219 (2016) 84–99. World association for the advancement of veterinary parasitology (WAAVP) guideline for testing the efficacy of ectoparasiticides for fish.

Golledge, H. and Richardson, C. (2024) The UFAW Handbook on the Care and Management of Laboratory and Other Research Animals. 9th edn. Wiley.

We will compare and utilise the experience of other scientists in the field e.g. from published work on sea lice vaccination (e.g. Casuso et al., 2022, Exploring Sea Lice Vaccines against Early Stages of Infestation in Atlantic Salmon (*Salmo salar*), doi: 10.3390/vaccines10071063)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly check updates to the regulatory guidance for testing veterinary medicines from the UK, US and European Union to ensure that studies meet requirements. We will use the most refined methods for testing veterinary medicines to meet UK or EU regulatory requirements, as defined by the Veterinary Medicines Directorate (VMD) and the European Medicines Agency (EMA) respectively, both of whom promote the 3R's and use of non-animal technologies. We will seek prospective authorisation for other testing e.g., Food and Drug Administration (FDA).

In addition, we will regularly check for updates to the recommendations included in the ARRIVE 2.0 and PREPARE guidelines.

We will encourage personal licence holders to open a N3CR's Experimental Design Assistant account and attend a Virtual demonstration to facilitate good experimental design when planning animal trials.

We will subscribe to the N3CRs newsletter, keep updated with developments through 'Understanding Animal Research' and attend RSPCA meetings whenever opportunities arise.

78. Immune control of parasite infection and tissue injury

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

parasites, Tissue repair, Infection, Allergy, Scarring

Animal types	Life stages
Rats	Adult
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult
Gerbils	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 'type 2' arm of the immune system is important for fighting infection with parasitic worms but is also involved in reducing inflammation and repairing damaged tissues. The aim of this project is to understand how ‘type 2’ immunity performs these different but related tasks.

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?

Type 2 immunity is essential to maintain tissue health in the face of infection with large tissue-migrating parasitic worms. Theses immune pathways are needed both to repair the damage caused by these worms and to reduce parasite numbers. Type 2 immunity is also involved in tissue repair more generally influencing how our bodies produce and lay down

the molecules outside our cells that give our bodies structure, elasticity and hydration (the extra-cellular matrix). However, when these pathways become dysregulated they can lead to allergy, chronic diseases and tissue scarring. Learning about these immune pathways is relevant to understanding many non-parasitic diseases that involve the type 2 immune response such as asthma.

What outputs do you think you will see at the end of this project?

Outputs will include:

A greater understanding of how tissue repair pathways interact with the immune system to cause or prevent disease, and critically how this immune information is communicated between organs in the body.

Publications disseminating our findings to global scientists.

Increased collaboration with clinicians on the use of drugs that block type 2 immunity

Who or what will benefit from these outputs, and how?

Short term benefits will be to other medical researchers who study parasitic worm infection and wound repair. Within the five years of the project, clinicians currently using drugs that block type 2 immunity could have a better understanding of their potential use and limitations. We also anticipate a greater understanding of the immunology of the body cavities that surround our intestines and lungs.

Mid to long term benefits will be to enhance our understanding of existing treatments for allergic diseases. These include drugs such as dupilumab, which is directly relevant to this project and is increasingly used in the clinic. Our work may lead to broader potential uses for these drugs, or a greater understanding of their potential limitations. Our work will also contribute to the development of new potential therapies designed to target the extra-cellular matrix. Our work to study how the immune system communicates between organs in our body has the potential to enhance understanding of why some people have disease symptoms in more than one tissue. For example, we may discover pathways that link allergic skin disease to lung diseases such as asthma. We also anticipate helping the development of ways to prevent or treat diseases of the body cavity, such as scarring that follows abdominal surgery, or how cancer cells can use these cavities to move around the body.

How will you look to maximise the outputs of this work?

To ensure maximum dissemination, we aim to publish in journals that reach a wide, and if possible, interdisciplinary audience. Furthermore, we place the first drafts of our published data on open access repositories such as www.biorxiv.org. To prevent unnecessary repetition of experiments by others, we seek to publish all data generated under this project including negative results.

Communication will also be by presentation at local, national and international congresses, where we regularly present unpublished data. We also talk regularly to clinicians at these meetings. In addition, our recent funding includes co-investigators who are clinicians. To enable rapid translation of our findings to the clinic we will exploit new and existing collaborations taking advantage of the University support toward translation. Importantly, all our studies are highly collaborative ensuring communication across different research groups.

We also actively engage with the public, in particular, we have media training and talk to the media (e.g. radio interview) about recent cases of parasitic infection in the news.

Species and numbers of animals expected to be used

- Mice: 25,000
- Rats: 240
- Gerbils: 120

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 versatile and tractable model for establishing models of immune pathology and extracellular matrix remodelling. The immune system and matrix components have been extensively described in mice and continue to provide insight into their importance during asthma pathology, viral and parasite infections in humans. Furthermore, the availability of immunological/matrix reagents and transgenic lines for mice, allows for approaches to manipulate immune-matrix components that delineate multi-factorial cell-cell and cell-matrix interactions that would not be possible using other species.

We will generally use adult mice in our experiments (>6-8 weeks of age), as at this age the mouse's immune system has developed to a degree that models the adult human immune system. However, important data suggest that modulation of the immune system in early life can alter susceptibility to external insults such as infection. Therefore, in some experiments we will use neonatal or juvenile mice to try and uncover important ways in which alterations in the immune system during early life can have important effects on the immune system.

Gerbils are the only suitable laboratory rodent capable of generating sufficient numbers of the blood circulating stage to allow transmission to the mite vector. Gerbils are thus essential for lifecycle maintenance, but do not have the genetic or immunological tools needed for detailed research. Mouse pups (typically 6-10 days old) are needed to maintain the mite vector, which transmits infective larval stage of the filarial parasite. Rats are required to generate infective larvae of the hookworm parasite we study.

Typically, what will be done to an animal used in your project?

Typically, mice will be infected with parasites, viruses, and/or be exposed to an allergen, and/or receive a single or multiple injections containing immunomodulatory substances (e.g. antibodies to neutralise a specific immune mediator or deplete a specific cell type, or cells to promote a particular response).

Experiments might look at the immediate immune response in the first few days after infection, allergen exposure, or administration of an immunomodulatory substance, or may last several months to allow full response development, or assessment of immune memory. These experiments will typically last between 1 day and 3 months. For longer experiments, mice may receive multiple doses of an immunomodulatory substance (e.g. once a week for 2-3 weeks), and samples (e.g. blood) may be taken to monitor immune response development over time. Experiments will end with animals being killed humanely, sometimes under terminal anaesthesia.

The cumulative experience of mice will typically be exposure to 2 or 3 procedures that may each cause short but usually separated periods of typically mild or potentially moderate degrees of suffering.

Separate from the above experiments, some genetically altered animals will be used only to breed and maintain animal lines.

Gerbils are typically infected with parasites, and then after several weeks (when parasites are producing offspring), they will be exposed to mites. The exposure to mites will typically occur 3-5 times and typically 2 weeks a part.

Rats are typically infected with parasites and kept for 8 days while they are generating eggs in their faeces.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of the animals undergoing experimental procedures, even with immunological manipulation, will experience mild, and no more than moderate, severity limits.

Breeding and maintenance of genetically altered mice with specific deletions in immune function genes or transgenic expression of immune receptors are not expected to exhibit any harmful phenotype. Our assessment of damage and repair due to parasite migration, viral infection or allergen challenge can result in local or systemic inflammation and pathology. This can manifest as weight loss, the involuntary bristling of fur, reduced spontaneous activity and reduced response to external stimuli. However, in most cases, only a small proportion of experimental animals will develop beyond mild symptoms to moderate severity limits. In investigating lung inflammation by airway manipulation using established models, some animals (5-10%) may experience temporary (less than 24 hours) respiratory symptoms resulting in moderate severity limits. However, most experimental animals will not develop beyond mild symptoms. Some protocols will involve general procedures such as restraint, injection or use of anaesthesia. All of these provide the possibility of adverse effects, but none beyond moderate severity. All animals will be humanely killed at the end of each 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 project works to a maximum severity of moderate. Across the license, we expect the majority of mice, rats and gerbils undergoing experimental procedures to experience mild (approximately 85%) severity, with <15 % experiencing moderate severity. Animals in breeding protocols should all experience 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 are studying complex processes that develop over time and trying to understand how the immune system handles these conditions and how different tissues communicate across the body to ensure that the right response occurs in the right place. All of these models involve the entire body, from the parasite infection model that involves migration of the worm from the skin to lung, to the viral infections which have systemic consequences, to the allergic airway remodelling that requires chronic exposure, and thus cannot be replicated in vitro. While experimental human studies of infection are sometimes preformed in very controlled settings, it is not possible to analyse the whole-body response in the different tissues. Similarly, the parasites that we study cannot be maintained in vitro and need a mammalian host to develop from the infective larval stage through to mature adulthood to mate and produce more parasites.

Which non-animal alternatives did you consider for use in this project?

Where it is appropriate we use in vitro systems to address particular problems. For example, we add immune factors to cell lines, to study the response in vitro. We have also worked with colleagues to add macrophages (a major immune cell of interest) to tissue organoids to understand the interaction of these cells with the tissue structure. Increasingly, we are using human tissue, for example from lung biopsies to assess the extra cellular matrix in the lungs of asthmatic vs non-asthmatics. Importantly, with more advanced technologies, particularly in computing, we are able to be move selective in the immune pathways we study, ensuring that what we are doing is the most relevant to human disease.

Why were they not suitable?

Our studies rely on looking at the immune response to infection or other challenges in the context of the whole body. We consider the consequences of exposure in both the local tissue (e.g. skin) and the impact in other distant tissues (e.g. lung). Many of our models involve long term infections, in which parasites migrate through the body and mature, with each parasite stage interacting with the host in distinct ways. Our allergy models look at the consequences of repeated exposure over time. Many of our studies consider the long term effects of exposure to allergens/parasites in the immune system later in life. None of these processes can be tested in vitro or with organoids. We are particularly interested in how immune cells interact with the extracellular matrix, which changes over time and in reponse to inflammation, a process that itself is poorly understood and cannot be replicated in vitro. We have also found that when we remove our immune cells of interest from the body, they lose many of their tissue-specific features, making the use of cell lines impractical.

Experiments to track immune cell interaction, activation and function over time are not possible with human samples, even where we have access to tissue, and we cannot experimentally manipulate humans.

Our research also depends on generating the infective stages of nematode parasites. There is no alternative means of generating this stage other than in a mammalian host (mice, rats or gerbils).

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 mice has been estimated based on experience gained under my previous Home Office licenses, taking into account breeding strategies for genetically altered mice, and anticipated numbers of planned studies over the course of the license.

Reflecting Replacement, Reduction and Refinement of our protocols over the past 5 years, we have reduced our anticipated numbers of animals for this application by around 40% from our previous project license.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We now purchase only the number of gerbils that we need from a commercial supplier, with a reduction in overall gerbil numbers compared to breeding in house.

Time mated female mice are bought in to supply pups for the mite feeds. The number of pups has been reduced to 9 per cage to ensure the pups are the correct size to use in the mite feeds. Surplus pups are not killed but fostered onto females from which pups have been taken from previously. Some of these pups are used when they come of size. The animal facility also make use of the foster mums for any pups from other parts of the animal facility.

For all of our mouse experiments, in-bred mice are used to reduce experimental variation, which makes it possible to use fewer animals to achieve statistical significance. For the majority of our studies, mice from the same litters are used for control and experimental mice, reducing variation that can occur due to differences in the microbiota. Overall, our experiments are designed to reduce the number of variables (for example age) to as few as possible and thereby reduce the number of control groups required.

All lab members are introduced to the NC3Rs experimental design assistant and encouraged to use it. However, everyone in the lab is trained in statistical methods and these are regularly discussed at lab meeting. We sometimes use more rigid criteria than the NC3Rs assistant if all agree that it the most appropriate for the experiment in question. Randomisation and blinding are included whenever practically possible. Tissue-sharing is a major tool we use to reduce animal usage.

A significant proportion of our animal use is related to breeding programmes for genetically altered lines. We follow the advice of our animal facility staff to optimise breeding, and regularly discuss numbers at lab meeting to ensure we do not overbreed. Where possible and appropriate, we use substances that can target or block immune processes in wild type mice, to reduce use of genetically altered mice.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We actively discuss all experiments in advance with all members of the lab so as to avoid any duplication and allow lab members to share animals. Tissue sharing occurs not only within our own lab, by through active discussion with other research teams, where we have found opportunities to work together.

The increased use of genetically altered animals has led to more complicated breeding strategies and, as a result, larger colonies. We reduce the numbers of these animals in our experiments by using littermates as controls wherever possible. Indeed, by including the heterozygotes in our studies, we have made important discoveries on the impact of gene dose. Additionally, when a particular strain is not being used experimentally we work closely with the animal technicians to develop a breeding strategy that maintains low numbers of stock animals.

We also regularly monitor animal numbers at lab meeting to ensure they are being used when needed – and all lab members are aware of any available 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.

Litomosoides sigmodontis is a mouse model for human filarial disease, reflecting closely the life cycle timings in humans. By using different strains of mice we can model both susceptibility and resistance to disease, as well as key features of immune regulation. A limitation of this model is that it does not reflect pathology to the disease, but the result is that we can study immune responses and parasite control with minimal animal suffering. In addition, this is the only model of type 2 immunity in the fluidfilled cavity that surrounds the lung, a tissue that is very understudied but relevant to multiple human diseases.

Nippostrongylus brasiliensis provides an excellent model of tissue repair in the lung. The migration of the parasite through the lung induces a classic inflammatory response, followed by an appropriate type 2-mediated repair response. This allows us to study injury repair pathways and by adjusting the dose we are able to minimise animal suffering but still study the pathways of immune cells and the remodelling of tissue structures that lead to effective repair. We compare these to models where repair is less efficient such as following flu infection, or following chronic allergen exposure. *H. polygyrus* and *T. muris* are natural parasites of mice that have a direct fecal-oral route and allow us to study parasites that only infect that gastrointestinal tract and which cause minimal adverse effects.

Mice represent the most appropriate species for in vivo study of immunity, because of the extensive knowledge of their physiology as it relates to humans, the genetic and biological tools available and the ability to be easily bred and handled. We will only use mouse models that have been refined to minimise animal distress. Procedures involve administration of virus or allergens by inhalation, or parasites by injection or direct delivery, and injection of immune modulators to reduce or alter disease progression.

Most of the animals undergoing experimental procedures, even with immunological manipulation, will experience mild, and no more than moderate, severity limits. Doses and timing are carefully managed such that animals will experience minimal suffering. We are constantly assessing and refining our approaches to ensure robust experimental results whilst minimising pain, suffering or distress.

We have recently acquired genetically modified mice that do not necessitate us to perform whole-body irradiation for the study of certain genes.

Why can’t you use animals that are less sentient?

Standard laboratory mouse strains are readily infected with the pathogens or allergens we study via the natural route (e.g. via the nose). The route of infection, site of pathogen development and the immune cell response are similar to that in man. Unfortunately, none of this can be replicated in less sentient species. To our knowledge, no other species of lesser sentience can fulfil the requirements of this project to the same extent as the laboratory mouse. We are studying long and complex immune processes and trying to understand how different different parts of the immune system communicate with the body to orchestrate an appropriate response. For example, we need to understand how cells communicate with the extra-cellular matrix, a scaffolding made of large proteins and other molecules which provides structure, holds cells together, and helps them communicate. The extra-cellular matrix components that regulate immunity are highly conserved between mouse and man, but would be very different in a non-mammalian species. We cannot use terminally anaesthetised animals as we require the mice to develop immune responses to the external challenges, and to analyse the outcomes of these challenges over time and in response to interventions.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All procedures will be performed by trained and skilled personal licence holders, who will handle animals with care. Animals will be monitored for adverse effects using score sheets previously developed in conjunction with the NVS and NACWO. These score sheets have proven to allow for objective measurements of clinical signs associated with adverse effects to determine when humane endpoints have been reached.

In line with the establishment's policy, we will adopt the latest techniques in animal handling (e.g.

cupping) to significantly reduce the stress associated with procedures. Furthermore, where possible, the least invasive methods for dosing and sampling will be applied.

Anaesthesia and analgesia will be provided where suitable (e.g. for humane restraint, during or in recovery from surgery). Antibiotic cover will be used to prevent opportunistic infections after irradiation.

We are constantly considering potential refinements to improve animal welfare including enrichment. For example, gerbils are prone to seizures when stressed. Our team began to reward them with sunflower seeds each time they are handled for a cage change or blood sample. The seizures are now very rare, and gerbils exhibit positive behaviour when the technician approaches in anticipation of the seeds.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The government animal testing and research: guidance for the regulated community

(https://www.gov.uk/guidance/research-and-testing-using-animals)

Morton et al 2001, Refining procedures for the administration of substances; Laboratory Animals, 35, 1-41

The NC3Rs webpage: https://www.nc3rs.org.uk/

The PREPARE and ARRIVE guidelines: https://arriveguidelines.org, https://norecopa.no/PREPARE

Standard Operating Procedures developed with the animal facility and the named veterinary surgeon.

For specific models, we read papers from other groups doing similar experiments, as well as consulting directly with other researchers to discuss the most refined procedures.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our team consult closely with the animal facility and take full advantage of the extensive resources provided to ensure we are following current best practices. We will continue to work closely with our local 3Rs representative to ensure we stay informed about the advances in the 3Rs. For example, we attend Experimental Design workshops to ensure we achieve our scientific aims with the minimal number of mice.

79. Hyperpolarised 129Xe Nuclear Magnetic Resonance (NMR) Spectroscopic Brain Oximetry

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

neuroimaging, brain, oxygen, MRI, ageing

Animal types	Life stages
Rats	Adult
Mice	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 is to develop and validate methods to measure oxygen in the human brain using MRI scanners.

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 has been known for some time that the amount of blood reaching the brain declines as people get older, but scientists do not understand whether this means the brain struggles to get enough oxygen. The aim of this research to study MRI brain imaging methods, to determine whether the amount of oxygen delivered to the brain can be measured non invasively. If so, doctors could send middle aged people for a brain scan to see if their brain is getting enough oxygen and this could help doctors identify people at risk of

developing dementia. There are various medicines and lifestyle factors that could be prescribed to boost blood flow and oxygen to the brain of such patients.

What outputs do you think you will see at the end of this project?

The output will be a systematic understanding of measurements from the brain using an MRI method and the inhalation of a gas. The gas can be thought of like a dye, which is detected by the MRI scanner. It is inhaled by the person and travels to the brain via the bloodstream just like oxygen. By measuring how readily the gas passes from the blood stream into the brain tissue, I intend to gain insight into how oxygen is reaching the brain. The gas is already being used with MRI scanners to diagnose lung diseases so we know it is safe and effective for humans. I output of this work may be that this gas can be used with MRI scanners to diagnose brain diseases. This could help detect dementia earlier, but it could also help doctors understand the ageing brain and how cardiovascular health is connected with brain health. The outputs will definitely include scientific publications and may include patents and a new diagnostic approach.

Who or what will benefit from these outputs, and how?

Patients are likely to benefit from these outputs. As societies age the economic burden of elderly people suffering from dementia is high. Early detection of dementia can lead to disease modifying interventions in midlife which prolong the onset of dementia, improve quality of life for the elderly and reduce the cost of care for the elderly.

The short term scientific benefit is understanding the method available to clinical researchers. While many new MRI methods arise, few transition to widespread clinical use. Significant evidence from translational research, using animals, healthy human volunteers, inanimate phantoms, is required to convince radiologists to implement a new method. Decisions are reached based on cost, ease of use and patient benefit far beyond the scope of this research. But if we consider the expanding use of hyperpolarised xenon to monitor the progression of chronic lung diseases such as idiopathic pulmonary fibrosis and chronic pulmonary obstructive disease, there is evidence that advanced MRI methods benefit diagnosis, treatment plans and patient management. The short term scientific benefit here is to test the oxygen sensitivity of this method to offer new insight into brain physiology and deficit of gas exchange in the brain. This will inform future decisions about the use of this MRI method in humans and patients.

How will you look to maximise the outputs of this work?

I am collaborating with world leading experts in MRI, brain and cardiovascular physiology. I will publish my research in prestigious, well read scientific journals. I will present my research at international scientific conferences. There may be opportunities in the future to collaborate with clinical scientists.

Species and numbers of animals expected to be used

- Rats: 360
- Mice: 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.

Mice and rats are the smallest and least sentient animals that mimic the elements of mammalian physiology under scrutiny here. The animals are small which minimises the cost of housing, and makes best use of the research funding. With regard to life stages, male and female adult rats will be used to harmonise data in terms of brain size and brain physiology. With regard to mice a group of male and female mice will undergo brain scans throughout their lifetime to understand how their brain physiology changes as they age.

Typically, what will be done to an animal used in your project?

Rats: The adult rat will be placed in a box with an anaesthetic gas which causes the animal to fall asleep. Once deeply anaesthetised the animal will be placed on a warm bed and the gas which maintains anaesthesia will be delivered via a face mask. An incision will be made in the lower abdomen and two plastic tubes will be inserted in the vein and artery above the leg. A second anaesthetic will be administered via the plastic tube in the vein. The face mask delivering the gas will be removed. A small plastic tube will be inserted into the rats wind pipe. A small hole will be drilled in the rats skull and an optic fibre to measure oxygen will be inserted 2mm into the brain. The rat will be moved into the MRI scanner. During the MRI scan: 1) the rat's body temperature will be measured and controlled by a warm water bed; 2) the rat will be ventilated to control and measure the breathing rate; 3) mean arterial blood pressure will be measure via the small plastic tube inserted in the artery. During the MRI scan the rat will be subjected to low oxygen conditions. This means the amount of oxygen in the gas the animal is breathing will be intentionally lowered. The low oxygen condition will be monitored via the blood pressure and the optic fibre in the brain. MRI scans will occur during low oxygen, then the oxygen level will be returned to normal. The animal is unconscious throughout and is killed at the end of the experiment, it never regains consciousness from the initial anaesthetic gas.

Mice: Human studies have shown that haemoglobin in the blood interacts with the xenon gas and this is how the MRI measurement changes when the xenon has moved from the blood stream into brain. Unfortunately this effect is not preserved in mice because their haemoglobin is quite different to human haemoglobin. To overcome this barrier, I will use transgenic mice that has been engineered to make human haemoglobin. There will be two groups of 25 mice, 50 in total. The first group (n=25) will be a control group and fed a healthy diet. The second group (n=25) mice will be given an injection to make them develop atherosclerosis and will be fed a high salt, high fat diet. On the day of an MRI session, the mice will be injected with a sedative, just under the skin. Once under sedation mice will be placed in the MRI scanner. After the MRI scan, mice will be kept in a warm chamber until they have recovered from the sedative. The mice will be scanned 3-4 times during the course of their life which is expected to be 2 years.

After the MRI study the aged mice may undergo optical imaging under terminal anaesthesia. Once the mice are deeply anaesthetised the animal will be placed on a warm bed and breathing rate and body temperature will be monitored throughout. A portion of the skull will be removed so that a two photon microscope can be used to take pictures and videos of the cerebral blood vessels and surrounding brain cells. This data can be compared to the MRI data acquired through the animals lifetime. The animal is unconscious throughout and is killed at the end of the experiment, it never regains consciousness from the initial anaesthetic.

What are the expected impacts and/or adverse effects for the animals during your project?

Rats: No adverse effects are expected except for premature death. For example, should there be an experimental error in the dose of anaesthetic or the cannulation of arterial vessel the animal may die prior to the completion of the experiment.

Mice: The mice will awaken from sedation after the MRI scan. They may experience pain from the injection which should subside within 24 hours. As the mice age there is the possibility of age related illnesses. The mice that develop atherosclerosis are given a high fat diet which can increase the probability of skin 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)?

For rats the severity would be classified as non-recovery. After the animals become anaesthetised, they never regain consciousness.

For mice the severity would be classified as moderate. Half of the animals will develop atherosclerosis and may become overweight, this will not harm the animals and does not constitute suffering. The mice will be sedated by an injection and will have an MRI scan, this protocol is deemed mild since the animals will return to normal rapidly after the sedation wears off. However the culmination of atherosclerosis and repeated MRI scans constitutes a moderate severity rating.

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?

This project is about oxygen homeostasis in the mammalian brain which is governed by a coordination of the cardiac, respiratory and neurological physiology. Whole animals are required because they have all of these components (cardiac, respiratory and neuro systems) and they mimic the human brain.

Given the complexity of the mammalian brain, it is not possible to replace animals for these studies.

Which non-animal alternatives did you consider for use in this project?

I have searched pubmed using the search terms 'brain', 'hypoxia' and 'methods'. There are few noninvasive methods to measure gas exchange of hypoxia in the mammalian brain. The factors which effect hypoxia in the mammalian brain are complex and integrated eg. respiratory and cardiovascular physiology, arterial blood pressure, intracranial pressure, cerebral pulse pressure and more. I hypothesise that hyperpolarised xenon can be used to quantify both gas exchange and brain hypoxia, however the only way to test this hypothesis is to create conditions of hypoxia in a mammals brain. Non mammals such as fruit flies or zebrafish do not accurately reflect the conditions of the mammalian brain.

Quantitative mathematical models have been created by collaborators based on data acquired in humans. Modelling data and human data will be consulted and referenced throughout this project.

Data acquired from living animals may be used to update or create new models. Human MRI data will be acquired in conjunction with the animal data. Comparisons will be made and all experiments which can, will be conducted in human subjects.

I have consulted pre-existing data as an alternative, however the experiments proposed here are entirely novel.

Why were they not suitable?

The blood flow to the brain and the rate of gas exchange is in constant state of flux specifically with regard to oxygen use and carbon dioxide production. The partial pressure of oxygen and carbon dioxide in the arterial blood is determined by the lungs. For the purpose of this project it is essential to control the output of the lungs (with ventilation and gas challenges) and it is essential to measure oxygen in the brain during the MRI brain scans. The project seeks to correlate the brain scan measurements with the amount of oxygen in the brain. Experiments in whole animals with lungs, heart and brain working together, are required to prove how the measurements compare.

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 rats is calculated based on many factors outlined below:

The experiments have been designed to capture the maximum amount of data from a single animal, furthermore there will be within animal comparisons eg. data in the same animal will be compared when acquired during normal oxygen conditions and low oxygen conditions. However there is there are many factors of these experiments which cannot be predicted at this stage. The effect size is unknown which means the use of power calculations has been scarce. Because there are many surgical steps there is a chance that human error will result in some experiments being terminated before data collection has begun or completed. Another unpredictable factor is equipment malfunction. The experiment requires the use of many machines, and should one machine malfunction this could ruin an experiment. Another factor is MRI methods development. It is possible I will have to adapt and improve the MRI acquisition methods which involves some trial and error. If the core hypothesis regarding brain oxygen is incorrect a new hypothesis regarding blood brain barrier may be developed. Finally this number represents the maximum number of MRI experiments that may be conducted by myself or my staff over a period of 5 years. It is likely less animals will be used once research aims are met, this number represents the maximum and accounts for any perceived pit falls.

The number of mice is calculated based on some practical factors and some experience of comparing two groups of animals. Ordinarily data from 6-9 mice should be sufficient to demonstrate a significant effect that would be clinically useful should this method be applied in clinical trials. The longitudinal imaging data will take over 2 years to acquire which is expensive and time consuming. If there were to be any issues such as equipment malfunction or human error performing the experiments to few mice could be a waste of time and resources. Should any animals need to be culled for reasons unrelated to the research such as skin disease, if the experiment was only conducted with 9 mice, there

would be insufficient animals to report the data. Therefore I have calculated 25 mice per group to ensure adequate data is collected to warrant this use of time and resources. These mice will be staggered in groups of 8 and once the experimental aims are met the experiments will end. This means less mice may be used, this number represents the maximum number of mice.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

I will train my staff/students on cadavers, I will supervise my staff/students throughout their experiments until they are deemed competent. I will use blinding and randomisation 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?

Pilot studies and regular testing of equipment.

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.

Non recovery experiments in rats should cause minimal pain and suffering since the animal never regains consciousness.

The mouse model of atherosclerosis has been chosen because it causes thickening of the artery wall. Gas exchange of oxygen and carbon dioxide occur at the capillary bed, but oxygen can also diffuse from arteries and arterioles. The vessel wall (surface area and thickness) are factors which effect the rate at which oxygen can diffuse into the tissue. There is evidence that atherosclerosis makes the brain low in oxygen. This mouse model should illustrate a difference between a normally ageing mouse brain and an ageing mouse brain which has changes in the rate of blood flow and the rate of oxygen delivery. The hypothesis of this research is that hyperpolarised xenon MRI can detect these changes. It is hypothesised that these changes will be non existent when the animals are young (6months), subtle when the animals are middle aged (12months) and large when the animals are aged (18months).

The experiments with mice have been designed to minimise pain and suffering. For example, I would favour the use of an injection under the skin (sub cutaneous) which I consider less painful than an injection into the abdomen (intra peritoneal). However I would seek advice here from the vet to ensure my methods cause the least pain and suffering.

For mice that undergo recovery surgery for optical imaging, I will follow existing protocols which have been developed by other researchers to manage post-operative pain and stress during imaging.

Why can’t you use animals that are less sentient?

I am using rats that have been terminally anaesthetised. To assess the clinical sensitivity of the MRI method under scrutiny, comparing mice with and without atherosclerosis is essential. I have to use these mice at various stages of life because there is evidence that oxygen homeostasis in the brain worsens as mammals age. Assessing the sensitivity of the method means comparing data when the brains are very similar (when the mice are young), and as they become much more different as the mice get older.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

I will develop the procedures alongside trained vets. I will seek guidance and advice about how to best manage analgesia, stress or physiological monitoring during MRI scans.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

PREPARE guidelines

LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgey

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Attend all training provided by the University of Sheffield. Visit the NC3Rs website regularly. Discussion with colleagues.

80. The role of DNA Damage response and replication factors in genome stability and physiology

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

DNA Damage response, Genomic stability, Tumorigenesis, Cancer therapy

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 overarching aim of this project is to further elucidate the role of the DNA Damage Response (DDR) genes in/during tumourigenesis and their broader impact on other diseases and phenotypes known to develop in case of genomic instability, including immunodeficiency, neurological disorders, and infertility. Specifically, we seek to identify which DDR genes contribute/promote or suppress tumourigenesis and explore potential therapeutic strategies to counteract the development of these tumours.

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 DNA Damage Response (DDR) encompasses multiple pathways that are essential for repairing DNA lesions, such as double-strand breaks, damaged bases, impaired replication, and other genomic insults. The DDR is vital for maintaining genomic stability in healthy cells, thereby preventing tumour development and other diseases. In humans, inherited mutations in DDR genes are linked to a variety of disorders, including cancer susceptibility, neurodegeneration, immunodeficiency, and infertility. Moreover, the DDR is frequently physiologically deregulated in many cancers, increasing cells' vulnerability to DNA damage and making them more dependent on alternative pathways for survival.

Targeting the DDR is a promising therapeutic strategy for cancer treatment, as it could be used to selectively kill cancer cells. Further exploration of the DDR could uncover previously unrecognized vulnerabilities that may be targeted in cancer therapy. Studying the DDR in animal models will also enhance our understanding of its roles in other physiological processes such as development, immunity, fertility, and neurophysiology.

What outputs do you think you will see at the end of this project?

My lab focuses on understanding how DNA Damage Response (DDR) pathways, including homologous recombination (HR), contribute to organismal homeostasis and disease. Since DDR processes are often inactivated in hereditary human diseases and are frequently disrupted at the somatic level in various cancers, we aim to investigate how these pathways influence human pathologies such as cancer, immunodeficiency, accelerated aging, and fertility. By leveraging the unique experimental strengths of C. elegans worms, cell-free extracts of frog eggs, and mammalian cell culture, my lab has identified new DNA repair genes relevant to human disease. This project seeks to determine how these disease-relevant genes contribute to genome stability, immunodeficiency, aging, and cancer through mouse models. Our research also has the potential to uncover new opportunities for targeted cancer therapies, as well as therapies against other human syndromes affecting fertility, immunity, and development, which we will further explore in mice.

We expect our work to be published into scientific journals to be made available to the whole scientific community and has the potential to open new drug discovery avenues for targeting specific pathways in the DDR.

Who or what will benefit from these outputs, and how?

Our research aims to uncover fundamental insights into how cells and entire organisms repair DNA damage to preserve genomic integrity, and how the failure to repair DNA can contribute to cancer development and other diseases, including immunodeficiency, infertility, and accelerated aging. The questions we address are highly relevant to clinical outcomes, as unrepaired or improperly repaired DNA damage is recognized as a critical early step in carcinogenesis and impacts germ cell development and immune function. The data generated from this project will not only deepen our understanding of DNA repair mechanisms and their role in maintaining organismal homeostasis which will benefit the whole scientific community through data publication and also guide the development of targeted therapeutics in collaboration with pharmaceutical companies.

How will you look to maximise the outputs of this work?

Findings will be made available to the broader scientific community through publication in peerreviewed journals and presentations in scientific conferences. Data will also be deposited onto publicly accessible databases. Newly created transgenic animals will be distributed widely to the scientific community as these lines generated under the authority of this Project Licence will be very valuable to the research community.

We may be collaborating with drug discovery organisations and pharmaceutical companies in order to initiate drug development and discovery on specific DDR targets that we are studying at the cellular and molecular level in the lab.

Species and numbers of animals expected to be used

- Mice: 10450

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 provide a robust and versatile model for studying tumourigenesis in the context of genomic instability due to the conservation of DDR pathways, the relevance of tumour development to human cancers, the ability to study complex interactions with the immune system, and the potential to test therapeutic strategies.

These key reasons are grounded in both biological relevance and experimental practicality:

Conservation of DDR Pathways: The DNA Damage Response (DDR) pathways that are central to maintaining genomic stability are highly conserved between mice and humans. Mice possess homologs for most human DDR genes, making them a valuable model for studying how disruptions in these pathways contribute to tumourigenesis.

Tumour Development Similar to Humans: Mice develop cancers that share significant molecular, histological, and progression similarities with human tumours. For example, mouse models of breast cancer, lung cancer, and colorectal cancer recapitulate the genetic and environmental factors involved in human carcinogenesis. This makes them ideal for studying the complex interplay between genomic instability and cancer development.

Functional Modelling of Human Cancer Genes: Mouse models allow for precise genetic manipulation, such as knockout or knock-in of specific DDR genes. This enables us to observe the direct impact of genomic instability on tumour formation and progression, providing insights into the role of specific DDR components in cancer. These in vivo studies cannot be replicated in simpler organisms like flies or worms, which do not develop cancers

Immune System and Tumour Microenvironment: Mice have an immune system that closely resembles that of humans, which is crucial for studying the interaction between tumours and the immune response. The immune microenvironment plays a significant role in tumour progression, and understanding this interaction requires a mammalian model with a fully functional immune system.

Relevance to Cancer Therapies: Mouse models are widely used to test the effectiveness of cancer therapies targeting DDR pathways, such as PARP inhibitors, which are designed to exploit the vulnerabilities in cancer cells with defective DNA repair mechanisms. The ability to test these therapies in an in vivo context where tumourigenesis and genomic instability can be studied together provides essential preclinical data before moving to human trials.

Phenotypic Outcomes Beyond Tumourigenesis: Mice models offer the opportunity to study broader phenotypic outcomes of DDR gene disruptions, such as immunodeficiency, infertility, and neurological defects, which often accompany genomic instability and cancer in humans. This multisystem impact cannot be fully understood using cell lines or simpler organisms, making mice indispensable for holistic studies of DDR-related pathologies.

We will be using all mouse life stages. Embryos will be required for generation of cells and studying the effect of a gene on development before birth. Animals from birth to late into

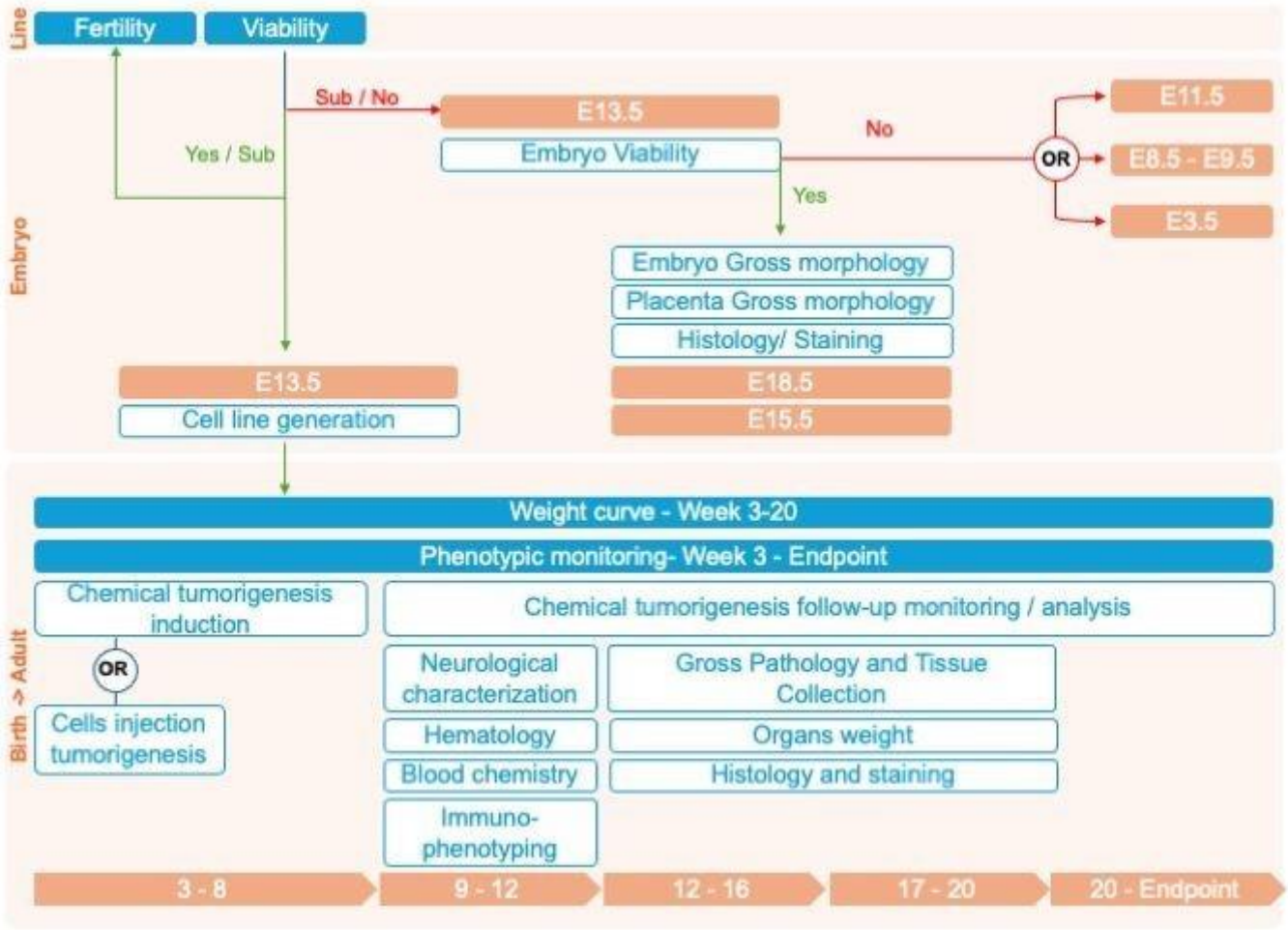
aging will be monitored in order to 1) look at tumour predisposition and/or disease development over the course of their lifetime, 2) examine grafted or chemically induced tumour formation/progression using well established tumourigenesis protocols targeting specific organs.

Where possible, we will replace animal experiments with cell culture experiments and the use of worm and/or cell-free frog extracts. In cases where this is not feasible, we will adhere to the principles of the Three Rs (Replacement, Reduction, and Refinement) to minimize both the number of animals used and their suffering during experiments.

Typically, what will be done to an animal used in your project?

Mice used will be generated to create conditional or constitutive knockout, knockin, expressing and/or over-expressing transgenic mice for genes we have previously implicated in the maintenance of genome stability or will be provided by other projects with authority to breed, maintain and/or supply transgenic mice.

Typically, genetically altered mice will be generally characterised following an adapted IMPRESS pipeline (see below).



These newly generated mice will be characterized by first looking at their viability. If they are viable, a clear pipeline will be followed starting with the analysis of fertility and the production of cells from either early embryos or mid-gestation embryos that will be studied in the lab. Mice will be subjected to frequent monitoring, including weekly weight checks and phenotypic observations. If they are not viable, we will perform an extensive study of embryogenesis with mid-gestation embryos harvest and analysis as a starting point.

Depending on the outcome, either younger or older embryos will be analysed to find out the last stages at which the embryos develop to.

To assess tumour predisposition in our genetically altered mice, we will employ four complementary approaches.

Groups of mice will be monitored in aging cohorts through frequent health checks, including clinical examinations, abdominal palpation, and non-invasive imaging when necessary to assess for spontaneous tumour formation. Moreover, these aging cohorts will give us insight into the broader physiology in which a specific DDR gene is involved and neurological, immunological and/or meiotic phenotypes of these mice will be examined through the course of their lives.

Breeding our mice with strains of mice known to develop spontaneous tumours with known onset and latency (for example p53 deletion or others characterised DDR mouse models) is a strategy that will be employed too. The time frame of this method will vary depending on the mouse model used to induce spontaneous tumour formation and it will be more controlled (time-wise and type of tumours forming) than approach 1. Information on onset and latency of the combined genetic alteration as well as tumour spectrum compared to the known mouse model will be evaluated.

Small cohort of animals will be treated with chemical carcinogens and/or UV/X-irradiation to specifically act as tumour inducers and/or promoters. Chemical carcinogenesis is one of the most wellestablished in vivo models for studying tumour development, as well as for evaluating tumour initiation, promotion, and progression. This approach will give us detailed information on the specific timing at which DDR gene are important during tumour development.

Transplantation of genetically altered cells (produced in the lab) into nude mice allow for the study of specific genetic mutations (knocking down or overexpressing certain DDR genes directly in specific cells type) and their impact on tumour development, progression, and metastasis. These tumours may be treated with potential therapeutic drugs and/or radiotherapy as a preclinical evaluation of potential patient treatment.

These complementary methods should enable us to determine whether a specific DDR gene plays a role in tumour formation and/or has a broader physiological role throughout the life of a mouse.

The duration of these experiments will vary depending on the approach taken to characterize a DDR gene going from 2-3 weeks for a xenograft experiment to a maximum of 2 years in the case of approach 1. We will always try to limit the duration of each experiment, and the number of animals used to address effectively scientific questions by taking into consideration which approaches are used (spontaneous versus induced tumours formation). Suffering will be kept to the lowest possible level by ensuring that animals bearing diseases are sacrificed in time to avoid distress.

What are the expected impacts and/or adverse effects for the animals during your project?

An important aspect of our research is to monitor animals that have genetic modifications throughout the term of their natural lifespan (up to 24 months) in order to study and assess their phenotype. Humane endpoints for mice used in such longevity studies have rarely been addressed, despite the expectation that health problems will become more common as the mice age. Natural and spontaneous death can occur without being linked to any genotype. In rare cases, aged animals can die without having shown any prior signs of

illness or deterioration in health. We will monitor animals at risk with greater than usual frequency and any changes in their health status will be accurately recorded on the animal database. If animals are found dead without having shown prior clinical signs, post-mortem investigation will aid determination of cause of death and this data will then be analysed as soon as possible so that early euthanasia can be performed on other animals to ensure their wellbeing as well as facilitating collection of valuable samples and measurements.

In general, mice are expected to develop tumours as adverse effects. The size and the impact of these tumours on the health of the animals will be different depending on the approach taken to characterise a specific DDR gene. Indeed, induction of tumours by cells transplantation may have a lower impact on the animal health as the tumours may stay external on the body but it will have a higher impact and cause more animal distress if cells develop into tumours that in turn spread through the body. It will depend on the type of cells used (aggressive versus slower growing) and also the site of injections. These models tend to be very well characterised and we can predict the latency of tumours formation taking into consideration parameters stated above.

Substance administration should not cause adverse effects that are longer than temporary and transitory as we will for most of them use doses that have already been described by the scientific community. If no data is available, we will perform small pilot experiments to determine the best regime to use in order to minimise adverse effects.

It is more difficult to predict adverse effects of newly created genetically altered animals and for this, general clinical signs of poor health will be used to assess mice condition such as weight loss, piloerection, hunched posture, refusal/ struggle to move, presence of wounds or ulceration, increased breathing. Health deterioration will be frequently check and animals will be immediately sacrificed if they appear to have more clinical signs of poor health.

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 will range from sub threshold to moderate. Indeed, we expect that a high proportion of mice (50%) should develop phenotypes of moderate severity as a result of tumour development, immunodeficiency and/or neurological problems.

Some experiments will have a more controlled endpoint (for example treatment with a drug causing no adverse effect) and clinical signs will not be required to sacrifice the animals (30%). These experiments will result in mild suffering.

A subset of these mice will also be used for breeding, tissue harvest and/or cell production with an expected severity to be subthreshold (20%).

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?

Mice provide a robust and versatile model for studying tumourigenesis in the context of genomic instability due to the conservation of DDR pathways, the relevance of tumour development to human cancers, the ability to study complex interactions with the immune system, and the potential to test therapeutic strategies.

Mice, unlike simpler organisms such as flies or worms, develop cancers that share significant molecular, histological, and progression similarities with human tumours. For example, mouse models of breast, lung and colorectal cancers recapitulate the genetic and environmental factors involved in human carcinogenesis which cannot be reproduced in 3D organoid models. This makes them ideal for studying the complex interplay between genomic instability and cancer development. They are a unique model allowing for precise genetic manipulation by deletion or overexpression of DDR genes to observe the direct impact of genomic instability on tumourigenesis and to test the effectiveness of cancer therapies targeting DDR pathways which can provide essential preclinical data before moving down closer to human trials.

Moreover, mice models offer the opportunity to study broader phenotypic outcomes of DDR gene disruptions, such as immunodeficiency, infertility, and neurological defects, which often accompany genomic instability and cancer in humans. This multi-system impact cannot be fully understood using cell lines or simpler organisms, making mice indispensable for studies of DDR-related pathologies.

Which non-animal alternatives did you consider for use in this project?

All of our proposed studies aim to build on extensive groundwork in model organisms, mammalian cell culture and during the last 15 years in mouse models. The objectives outlined in this application represent the next and most pertinent step forward in our mouse studies, which is likely to provide novel insights into the impact of these factors on organismal biology and disease. We are currently using a multi system approach to answer questions addressed in our studies. For example, we performed extensive pre-animal studies in mammalian cell culture, which was recently revolutionised by the discovery of CRISPR. Generation of genetically altered mouse or human cell lines is proving to be easier and quicker with CRISPR techniques and these cell lines can be used to do targeted genetic and proteomic screens that provide insight on molecular mechanisms that inform the study design for our animal work at a whole organism level.

Moreover we have been using and will continue searching the following resources in order to look for newly developed alternatives to in vivo work proposed in this project:

European Commission datasets on replacement of animals specifically in immuno-oncology

research, breast cancer alternatives and on alternatives to cell therapies

(https://jeodpp.jrc.ec.europa.eu/ftp/jrc-opendata/EURL-

ECVAM/datasets/DBALM/LATEST/online/dbalm.html)

3Rs resources on NC3Rs pages

NORECOPA database (https://norecopa.no/databases-guidelines)

Other publicly available 3Rs databases (for example: 3Rs Hub, https://www.3rsinfohub.de/organs/index.html)

Non-Animal Technologies Database (https://www.nat-database.org/)

We are avoiding duplication of in vivo work by performing extensive searches in scientific publication databases (such as PubMed, GoogleScholar, BioRxiv- preprint server) to ensure that the proposed work in this project has not been published yet. Moreover, attendance to various conferences within our field of interest and private communication with other world experts keep us up-to-date of the majority of new, non-published yet advances in our field.

Why were they not suitable?

While worms, cell culture and 3D organoid models are valuable research tools, they have significant limitations when it comes to studying tumourigenesis, particularly in the context of complex human cancers.

These models may not be suitable for many aspects of tumourigenesis research. C.elegans does not naturally develop tumours and the simple anatomy of the worm does not recapitulate the complexity of tissue-specific tumours. Similarly, organoids, which are 3D cultures derived from stem cells, provide a more advanced in vitro model than traditional 2D cell culture, allowing for the study of tissue-specific tumourigenesis. However, they do not replicate the full physiology of an organism and lack the complexity of an in vivo tumour micro-environment, including interactions with the immune system, vasculature, and surrounding stromal cells. This makes it difficult to study tumour progression, metastasis, and responses to different therapies.

While computer models provide valuable insights into biological systems and are useful for early-stage research, they lack the full complexity of in vivo systems. In vivo research remains necessary for capturing the dynamics of biological interactions, treatment responses, and toxicity concerns that are challenging to replicate computationally. For accurate predictions and translational potential, in vivo work is often indispensable despite the increasing sophistication of computational 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 estimated the number of animals to be used based on our last 15 years of working with mice and also more refined projects which allowed us to greatly reduce animal numbers over the different PPL.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Mouse breeding experiments will be planned in consultation with animal facility and the basic principles of mouse breeding will be adhered to. Gestation times, weaning age and litter sizes will be used to calculate the numbers of mice required to optimise the size of the colony and experiments requirements. Mouse lines will be routinely maintained by keeping 2 breeding pairs. For crosses under analysis for specific phenotypes it is likely that 5-6 breeding pairs will be maintained for the duration of the experiment. However, every effort will be made to reduce the number of animals required for experiments, always keeping in mind that enough animals are used not to prejudice the generation of statistically relevant results. For exploratory (pilot) experiments the least number of animals will be used (usually 4-6 per genotype/experimental group) to provide a reasonable estimate of the numbers of animals needed to produce statistically relevant information in subsequent quantitative experiments. Where possible we will use data from published experiments to provide an estimate of the sample size.

Where necessary we will also consult the bioinformatics and biostatistics group at the Crick during the experimental design stage to ensure that the appropriate numbers of mice are used per experiment. We will also use the Experimental Design Assistant from NC3R to help us design our experiments (ensure the use of minimum number of animals consistent with our scientific objectives).

For multiple genetic modifications, we will investigate alternative methodologies to enable reduced numbers of animals to be used. This could include the use of viral delivery of the expression modifying genes and tetracycline activator via different routes (intratracheal, intraperitoneal, topical application). This would require two less genetic modifications to be present in the mice, so would greatly reduce the size of the breeding programme.

We will also reduce numbers of mice by cryopreserving sperm or embryos of mouse lines where no immediate experiments are planned.

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 always looking for ways to reduce the number of animals that we produce. Breeding efficiency is key and for strains that are known to have low breeding efficiency, we feed females with baby milk mash. This allows us to use less mice for breeding and also increases the chance to have a higher number of embryos/pups in some of our mice strains. Additionally, colony size will be under constant review to adapt to experimental needs and reduce wastage from overbreeding. Moreover, most of our xenograft studies start with pilot experiment using small animal cohorts that lead to a refinement in term of cell number injected, and/or doses used for specific compounds and give us insight into experiment duration; and where possible we will use a single group of animals as a control for several treatment groups.

Other ways in which we will aim to reduce mouse numbers will include freezing down genetically altered lines; deriving cell lines from mice for specific cell culture experiments; providing tissues from our mice to other labs.

The breeding of genetically altered mice will be reduced through collaborative access to strains.

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 our model of choice during this project. We will be generating genetically altered mice models that we will characterise spontaneous tumour development and any other diseases known to develop in mice exhibiting genomic instability (neurodegeneration, immunodeficiency and/or meotic phenotypes). Moreover, we will use known tumourigenesis mice models to cross with our newly generated mice to study DDR gene deficiency induced tumourigenesis in a more controlled way (e.g. known latency, onset and spectrum of tumours). We will prioritise shorter term experiments (e.g. induction of tumourigenesis by breeding, chemical treatment or cells transplantation) to minimise animal suffering but on some occasion, longer term experiments (aging) will be necessary in order to fully characterize a specific DDR gene. The use of pilot experiments will help us minimising the number of animals used and also the suffering of animals during experimental procedures.

Why can’t you use animals that are less sentient?

Mice and Human are biologically and genetically very similar. By using mice, we ensure that we can study the effect of a single specific genetic alteration (for example found in human breast cancer or any other disease that have a deregulated DNA Damage repair pathway) in a controlled organism. Moreover, spontaneous tumourigenesis resembling human tumorigenesis cannot be reproduced in other organisms (flies, worm, frog, cell culture) as they do not develop tumours, immune-deficiency or neurological problems. Where possible experiments on animals will be replaced with experiments on cultured cells and C.elegans as outlined above. We will first attempt to test our hypothesis in tissue culture with cells derived from these animals, but ultimately, it is experiments with animals that will have the biggest impact on human disease and new therapy findings. We will minimise the numbers of animal used and minimise the suffering of animals during experimental procedures.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will monitor animals at risk with greater than usual frequency and any changes in their health status will be accurately recorded in our animal database. If animals are found dead without having shown prior clinical signs, post-mortem investigation will aid determination of cause of death and this data will then be analysed as soon as possible so that early euthanasia can be performed on other animals to promote their wellbeing as well as facilitating collection of valuable samples and measurements. We will use palpation and imaging in order to limit adverse effects due to tumour development.

The weight of the animals going through a procedure will be monitored closely to ensure weight is stably increasing and is not affected by any procedure that a mouse went through.

Most of the protocols that we are using in this project are well established protocols that have been widely used by the scientific community. We will follow them closely by administering the recommended concentration of substances (such as chemical carcinogen, tumours initiator and promoter, IR/UV doses, antigen concentration).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

FELASA “Working Group on Pain and distress” and NCRI guidelines will be employed to aid in the assessment of pain and distress and used to determine the earliest endpoint possible to allow a valid scientific outcome.

When assessing tumour burden, the NCRI Guidelines for the Welfare and Use of Animals in Cancer Research and OBSERVE guidelines will be followed.

LASA Guidelines will be followed for Administration of substances.

In addition, we will follow the PREPARE guidelines when designing experiments and ARRIVE guidelines when publishing to allow better reproducibility and avoid work duplications.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are currently subscribing to the NC3Rs monthly newsletter which gives us information on any techniques’ improvement and 3R advances. We also keep updated using information from 3Rs infoHub (https://www.3rsinfohub.de/organs/index.html), Animal Research Nexus (https://webarchive.southampton.ac.uk/animalresearchnexus.org/), CAAT (Center for Alternatives to Animal Testing, https://caat.jhsph.edu/resources/)

We receive regular updates on advances and new techniques from our own establishment.

81. Regulation of Thrombus Formation

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 Disease and therapies, Clotting Cascade, Anticoagulation therapies, Bleeding, Anticlotting therapies

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 overall aim of the project is to investigate the cellular and molecular mechanisms involved in thrombus (blood clot) formation and its breakdown and ultimately to specifically block or modulate these mechanisms.

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 formation of blood clots, although essential for normal haemostasis, can pose a significant risk to health. Thrombus formation is a contributory factor in heart attacks, stroke and atrial fibrillation.

Conversely, too much clot breakdown can result in bleeding. It is therefore extremely important that the underlying mechanisms of clot formation and clot breakdown are better understood so that more effective therapies, and ultimately preventative therapies, can be

designed. However, key problems with current anti-clotting therapies is the increased risk of uncontrollable bleeding. This adds to the importance of understanding the intricacies of the formation of clots and their breakdown.

What outputs do you think you will see at the end of this project?

Through this Project Licence we will gain a greater understanding of the initiation, formation, structure and stability of thrombi and ways in which thrombus formation can be inhibited. The detail of mechanisms and molecules involved in specific aspects of thrombus formation and its breakdown can be used to develop small molecule inhibitors of these target molecules. The clinical benefits may be identification of novel pathways that can be targeted with therapeutics minimising risks associated (e.g.

bleeding) with current therapies; this could be through the development of new treatments for thrombosis that are directed at modulating the developing clot, and separately, agents that can stabilise the clot as is relevant in patients with acute bleeding.

Who or what will benefit from these outputs, and how?

It is anticipated that the information gained through this programme of work will be of benefit to the scientific community through peer review publication. Findings gained from the in vivo work detailed in this project, together with in vitro data obtained will provide the research community with insight into the role of specific molecules in thrombus formation and clot breakdown. They will also provide a greater understanding of alterations in function in clinically relevant mutations of these molecules.

Small molecules showing the greatest inhibition of thrombus formation in vivo will be sent for further testing with the aim of reaching human clinical trials. Separately, small molecules that protect the clot from being broken down will also be further developed towards reaching clinical trials to address acute bleeding in patients.

How will you look to maximise the outputs of this work?

Our studies are conducted alongside other collaborators who have successfully established channels to share new knowledge to further develop and refine in vivo and in vitro systems to study thrombus formation at the cellular level. We have developed potent compounds that selectively interact with the specific cellular structures.

Our research findings will be shared with the greater scientific community in the form of national and international conferences and workshops and also through peer-reviewed publication.

Species and numbers of animals expected to be used

- Mice: 1,850

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 will be used as models of thrombus formation are well established in this species. Also, selective blocking antibodies and genetically modified or mutant mice

relevant to these studies are available. We also know that human clotting factors and inhibitors have physiologically relevant actions in mice. In addition to this we intend to use genetically altered mice with specific clotting factor mutations to study their effects on clot development.

Typically, what will be done to an animal used in your project?

Test compounds will be administered orally, intravenously or by injection under the skin 2-6 hours as a pre-treatment (once per animal).

under terminal anaesthesia, test compounds and fluorescently labelled agents will be given intravenously (via the carotid artery).

Clotting will be induced in the femoral vein or the cremaster vessels chemically or by laser injury.

Images of the clot will be taken every 10 minutes for 1 hour.

At the end of imaging, whole blood will be taken for plasma samples.

Mouse will be humanely killed at the end of the procedure.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of the animals used will undergo non-recovery anaesthesia to minimise suffering. If recovery is necessary, all procedures will be carried out by competent personal licence holders using appropriate procedures and experiments will be terminated should any sign of suffering (likely to exceed mild serverity) be observed.

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 % of mice will be used under non-recovery anaesthesia.

10% of mice undergo a surgical procedure under general anaesthesia and allowed to recover for a period of up to 7 days before being used under non-recovery experiments. This is to study the development of clots and the test compounds over longer periods of time. During the recovery period, the health and welfare of mice will be regularly monitored by experienced personnel.

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 initial experiments will be carried out using ex vivo models of clot formation, the use of animals is required due to the complex nature of the clotting cascade; this requires multiple factors to be present simultaneously in a biological system to evaluate the complete effect.

Which non-animal alternatives did you consider for use in this project?

Computer modelling of the interactions between test compounds and the target sights of the clotting structures are carried out.

To mimic blood flow conditions and clot development in vivo, human blood assays are used to form clots and compounds tested to breakdown clots in vitro alongside this project.

Why were they not suitable?

In vitro studies, as mentioned, provide valuable information regarding the cellular components involved in the clotting cascade but do not capture the inherent complexity of organ systems. The cellular interactions and the biochemistry involved in metabolic breakdown, safety, toxicity, and efficacy of test compounds are better evaluated in a clinically relevant biological system.

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?

Having received advice from our statisticians we estimate that 6 animals will be required for each experiment based on our experience using this model. These calculations will be constantly reviewed after initial experiments are performed to ensure they are accurate. If required, additional advice on study design and subsequent data analysis will be sought for more complex studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Internal controls will be used whenever possible. For example, in time course experiments progression of thrombus formation will be compared to fluorescence within the vessel recorded prior to injury. Where internal controls are not an option, test animals will be compared with inbred age-, weight- and sex-matched controls to minimize variation.

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 studies such as clot lysis assays and compound profiling studies (to analyse target specificity, potency and toxicity) are carried out to identify the best candidates for in vivo use. This will reduce the number of compounds being put forward for in vivo analysis.

Small pilot studies will be carried out for each family of compounds. Superior compounds will be carried forward for dose response studies. This will ensure inferior compounds are not part of large scale investigations therefore reducing the number of animals 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 as models of thrombus formation as they are well established in this species. Also, selective blocking antibodies and genetically modified or mutant mice relevant to these studies are available. We also know that human clotting factors and inhibitors have physiologically relevant actions in mice. Non-recovery anaesthesia is used throughout our studies to reduce pain and suffering to the animals.

Why can't you use animals that are less sentient?

Cellular components of blood and clotting factors involved in the coagulation pathways in humans and mice are closely related. We have a well-established murine clot formation model which utilises mutant mice as well as specific clotting inhibitors which are not available or compatible in less sentient species. Scientific outcomes using mice in these studies will be of greater relevance with the aim of our findings leading to human clinical trials in the future.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Most experiments will be carried out under non-recovery anaesthesia minimising the welfare cost as much as possible.

Animals used in recovery experiments will receive the appropriate post-operative care (pain relief administered, kept incubated and softened food provided if necessary) to reduce pain and suffering and they will be regularly monitored until fully recovered.

Any animals failing to recover within 2-3 hours post op will be euthanised.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The principles in the EDA(experimental design assistant), PREPARE and LASA guidelines will be followed for up to date information on experimental design to use minimum animal numbers and also information on statistical analysis methods to reduce repeat experiments.

https://nc3rs.org.uk/our-portfolio/experimental-design-assistant-eda
https://norecopa.no/PREPARE http://www.lasa.co.uk/wp-content/uploads/2017/04/Aseptic-surgery-final.pdf

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We regularly attend NC3Rs workshops/symposia which provides an opportunity to discuss and learn about the latest developments and advances in experimental design.

Experimental design workshops are also held during our annual group meetings to keep up to date with latest advancements.

We will also ensure that we keep up with advances by reading new publications and keeping up-todate with the availability of new validated models.

82. Investigating new therapeutics for eczema

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
- 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

eczema, atopic dermatitis, skin, therapy

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?

Using a lab based skin model, we will first identify molecules that can inhibit one of the environmental triggers of eczema, which is derived from the bacterium Staphylococcus aureus that lives on skin. We have already shown that Staphylococcus aureus also causes eczema in mice. We will test these molecules on the skin of mice which have eczema, in order to identify new treatments that can be applied directly to skin.

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?

Eczema, or atopic dermatitis affects 11-20% of children worldwide and 25% of these will continue to be affected into adulthood. Symptoms range from mild to severe, with those most severely affected suffering significant reduction in quality of life. We still do not fully understand which molecules are responsible for triggering the disease. Recent studies

investigating the role of the skin microbiome have significantly advanced our understanding, and have the potential to identify targets for new therapies.

What outputs do you think you will see at the end of this project?

New information: These experiments will complement our lab studies which have identified two key pathways which inhibit one of the triggers of atopic dermatitis. The results of these animal studies will not only enhance our understanding of how the immune system kicks off the skin response, but also the molecules involved in reducing this response. Interestingly, the immune response in the skin is similar to that seen at other sites in response to some infections. We hope our observations will be translatable to these conditions.

Publications: We will publish our findings in peer reviewed journals to share our data both with dermatology and immunology experts. We are also funded to attend one international conference over the next two years where we will present our findings to a global audience.

Products: We are investigating known inhibitors of the pathways we have identified, in order to try and repurpose known drugs / small molecules. If this proves successful, our aim is to pursue further funding to test these products clinically.

Who or what will benefit from these outputs, and how?

Academic beneficiaries (short and long term): A better understanding of the most common triggers of eczema will lead to new molecular targets to inhibit this particular immune response. We hope to demonstrate inhibition of the key pathway involved in triggering allergic disease which will be translatable to future clinical study.

Clinical beneficiaries (longer term): We will be using already licenced drugs to test their ability to inhibit the pathway we have identified. We hope this will enable further funding towards clinical testing and application.

How will you look to maximise the outputs of this work?

These studies will be running in parallel with our collaborators who are studying an alternative model of atopic dermatitis. Importantly, these two models represent different cohorts of clinical patients. The first model is spontaneous and driven by the environment without a known genetic cause. The filaggrin model that will be housed at our establishment represents patients who have a genetic mutation in the skin barrier which significantly increases the risk of eczema. Given that we are funded for a multi-site study, we will be able to demonstrate reproducibility of any treatments in two different types of eczema model. This will add considerably to our findings.

We will publish our findings at both academic and clinical meetings as well as publishing in well respected academic journals making translation quicker.

Although we have a target pathway in mind, we will use complex tests to look at the overall effect of inhibitors on clinical severity as well as skin and blood immune cells. Should treatments not demonstrate inhibition of the immune response, this will still broaden our understanding of the pathways to disease and will provide additional therapeutic targets not only in skin, but potentially in other tissues as well.

All unprocessed data will be made available through publications, external requests and repositories such as FigShare.com which is fully supported by this institution.

Species and numbers of animals expected to be used

- Mice: Total number: 610.

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 already have data in a different mouse model of eczema which shows that a skin bacterium, Staphylococcus aureus, causes clinical disease. We have also shown that our immune molecule of interest, IL-33, is of key importance for triggering the disease. Therefore the mouse provides an ideal organism to test potential treatments for eczema which will inhibit the release of IL-33. We will be using mice that have a genetic mutation in a skin molecule called Filaggrin. In these mice, the skin barrier does not function as it should allowing bacterial proteins to cross the skin barrier and cause an immune response. Filaggrin mutations are found in 40% of children with eczema, so these mice will help us to directly translate our findings. We will use mice when they reach adulthood and their skin microbiome has established.

Typically, what will be done to an animal used in your project?

Mice will be kept in a specific pathogen free environment with sterile bedding and water to prevent spontaneous development of skin disease. At 6-8 weeks of age, once their hair has reached a 'resting' growth phase, we will shave a moderate area of the back of each mouse. Allergens will be painted onto the back of each mouse daily for 4 weeks to monitor any disease. Treatments to inhibit disease may or may not be administered at the same time. Mice will be weighed every 2-3 days, and the condition of the skin will be scored using various clinical measurements. The majority are non invasive (observation only). At weekly intervals we will measure the 'leakiness' of the skin (trans epidermal water loss) using a hand held device that is held to the skin for a short time. We will also swab the skin weekly for skin bacteria and will apply tape to the skin to collect cells. These are both non invasive and painless for the mice. Mice will be housed with their littermates whether they are genetically mutated or not. This will help us to control for any changes in the skin bacteria that may happen between different groups of mice. At the end of the 4 weeks, mice will be humanely killed and skin and blood will be collected for analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Although the filaggrin mice can begin to show signs of eczema throughout life and this can become severe, this is entirely due to allergens in the environment. By keeping these mice in a pathogen free environment and using sterile bedding we will minimise any adverse effects of the environment. This means that we can control when the mice will start to show signs of eczema and how severe the disease will be. We already have preliminary data from a different type of mouse to help us understand the parameters of these experiments. We also know that the appearance of eczema will not resolve on its own in these mice, so we will only keep the mice for 4 weeks of treatment. Although we will be using sterile bedding and water, there is a small possibility that eczematous skin may become infected. If this adversely affects an individual mouse, we will see a fall in body weight and change in behaviour. As the colony will be monitored daily this will be picked up quickly, in which case the mouse will be removed, housed singly and monitored for

recovery. Bedding will be changed throughout and littermates housed in a new cage. These mice do not have a defect in their immune responses, only in the skin barrier, so we will make every effort to ensure a skin infection does not spread. Should the eczema become severe, we have a very well defined clinical scoring system which includes appropriate 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)?

The control mice (without filaggrin gene mutations) have a fully functioning and intact skin barrier, so will likely respond to allergens on the skin with either no visible or very mild visible and / or behavioural effects.

The heterozygote mice (with a partial filaggrin mutation) reflect the majority of human eczema patients, and exhibit a partial defect in the skin barrier. We expect these mice to respond to allergens on the skin with a mild to moderate visible and/or behavioural effects. Similarly we expect any control mice that have had their skin barrier artificially disrupted to also respond to allergens with a mild visible and/or behavioural effects.

The homozygote mice (with a complete filaggrin mutation) reflect a smaller proportion of human eczema patients and have a loss of function of the filaggrin protein. Although there are other barrier proteins in skin that continue to function correctly, we expect these mice to respond to allergens on the skin with moderate visible eczema, some itching and redness which may become severe over time. However in the absence of sterile bedding, these mice do not exhibit severe eczema until 28 - 32 weeks of age. Therefore we expect under the design of our experiments, that the majority of the homozygote mice will still be in the moderate category at the end of the 12 week period.

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?

Eczema is a disease driven by the immune response to allergens on the skin. Importantly, the severity of disease is dependent not only on the number of immune cells, but also the type that enter the skin. This is determined outside of the skin site. In fact these immune cells come from the bone marrow via the blood system and cause inflammation. We need to use a whole animal to test whether the drugs we identify can prevent or change the influx of immune cells and reduce the severity of disease.

Which non-animal alternatives did you consider for use in this project?

We are using primary human skin cells prior to and alongside use of animal models in order to screen suitable drugs for clinical use. This ensures clinical relevance and

translation to human disease. We have also used human skin biopsies to study the pathway of IL-33 release.

Why were they not suitable?

Although these models have allowed us to study the direct effect of allergens on the skin cells themselves, and on the skin barrier, neither allow us to study immune cell influx into the skin. We know from current therapies for eczema, that the only way to achieve clinical benefit is to inhibit the immune response in the skin. A better understanding of this in response to allergens is needed.

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?

There are no reported issues with breeding of these mice aside from natural gender and genotype variation across litters. Numbers have been calculated based on previous data from our collaborators which showed that 100% of mice treated with bacterial or house dust mite allergen show an eczema phenotype. This is our primary outcome measure so we have calculated that 100% of treated mice will be used for analysis. This is highly likely given the skin barrier defect in these mice. We have included 10% contingency here to account for variation in genotype, unproductive mating, loss of pups and should mice become infected and therefore unable to be used for analysis. Numbers of animals have been discussed with a statistician.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Both males and females will be used in this project. These numbers account for mixed gender group sizes. Our preliminary data shows that both genders respond to allergens.

All genotypes will be used in experiments. Since human eczema patients include both homozygote and heterozygote filaggrin mutations, both these groups will inform human disease.

All drug treatments will be screened first in our human skin model in the lab. Our data has shown that the response of skin cells is the primary trigger which drives the immune response in the skin. By prescreening we will only test drugs that have a high chance of success.

By keeping the animals in a specific pathogen free environment with sterile bedding and water, we have very good control of the disease phenotype. By ensuring less variation in disease outcome, we can more precisely measure the effect of drug treatments on the skin response.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

As we are using a well categorised mouse genotype, there will be no need for extensive breeding of the mice. We will use mice as they become of age in small groups, thereby generating data at all stages of the project. The pooled analysis approach will allow us to build power as the project progresses, so that we do not use more mice than needed to demonstrate an effect. As outlined above, we will pre-screen treatments against our lab based model to identify a small number of candidates for testing, thereby only testing those with a high chance of success.

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.

40% of eczema patients have a skin barrier which doesn't work as it should due to a defective filaggrin protein. In this project we will use mice that have either a partial or complete lack of filaggrin in the skin in order for our findings to be translatable to human studies. Separately, control mice with intact filaggrin, but that have had their skin barrier artificially disrupted will also be studied.

The approach we will use will be to expose these mice to allergens on a small skin area. This will induce eczema in the treated mice, which will cause increased itchiness, redness of the skin and a rash like that seen in eczema patients. We will expose only the mouse back to allergens to reduce scratching and skin injury. As we are looking for new drugs to treat eczema, we have to induce eczema in these mice. However we will only do this for a maximum of 4 weeks. This will be enough for us to see if the drugs we test are worth investigating further.

Why can’t you use animals that are less sentient?

It is well documented that scratching behaviour contributes to the severity of eczema, in mice and humans. Itching is an important part of the immune response and reflects what happens in patients. We need the mice to be awake and behaving normally in order to reproduce human disease.

We will use adult mice because this is the time that their fur reaches a resting growth phase. We need to shave a small area of the back in order to expose the skin to allergens. If the hair is 'resting' there will be no need to repeat the shaving during the experiment. In this way we can easily observe the eczema and measure the skin function.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be monitored daily for clinical signs of eczema, and weighed every 2-3 days. Weekly clinical scores will be recorded to generate longitudinal data. Collectively this means that any adverse change in clinical score will be picked up quickly.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use published literature to identify best practice guidelines for epicutaneous administration of allergens and treatments. Many of the atopic dermatitis models use agents or methods to break down skin barrier before administration - we will do this in control mice to compare with the mice which already have a skin barrier defect. We will consult clinical guidelines, toxicity and side effects of candidate therapies in order to refine doses and identify candidates best placed for future clinical studies.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The establishment engages in Continued Professional Development for researchers which includes attendance at 3R related seminars. 3R focused seminars are also available through the NC3R website. Our lab will engage with the Research Group Tool which regularly assesses the lab approach to 3Rs. We adhere to the PREPARE guidelines for project design practice.

83. Understanding the molecular drivers of thoracic 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, lung, SOX2, therapy, prevention

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 the project is to understand the impact of the molecules and cells that drive cancer and then use this information to develop 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?

Cancer is a major burden on human health and survival. Lung cancers and cancers that have spread to the lung are a major cause of human suffering. The work described in this application is ultimately intended to reduce human suffering from lung cancer and cancers that have spread to/from the lung.

What outputs do you think you will see at the end of this project?

There is a major unmet need for novel approaches to prevent and treat cancer of the lung.

We will provide a greater depth of mechanistic understanding of the development of tumours in the lung. This will lead to sharing of datasets in publicly available data repositories and publications in the scientific literature and presentations at appropriate scientific conferences (Timeline 0-5 yrs).

We will develop and test new approaches to treating cancer in the lung using the mechanistic knowledge we generate (Timeline 0-5 yrs).

We will disseminate this information widely, including to academic and biotech/pharma collaborators with the aim of developing an evidence base and rationale for novel therapeutic approaches or combinations with an explicit view to building on the preclinical work to inform experimental medicine in innovative clinical trials.

Who or what will benefit from these outputs, and how?

The scientific and biotech/pharma community will benefit from this work in the short and medium term (0-7 years). Ultimately it is hoped that patients and their families will benefit from this work in the longer term (Timeline >7years).

The scientific community will benefit from a more in depth understanding of the molecular mechanisms underlying cancer and the ability of cancer to form tumours in the lung (0-5 years). This will allow us and the scientific and biotech/pharma community to consider and test preclinically new therapeutic approaches to human cancers in the lung (Timeline 3-7 years). A key purpose of undertaking these experiments is that we will be able to develop new strategies to treating lung cancer and then, via collaborative approaches go on to test these in clinical trials (Timeline > 7years). In this respect we will seek to build an evidence base for traditional systemic delivery of therapeutics and also novel local therapeutics delivered directly to the lung cancer thereby avoiding systemic toxicity.

The scientific/biotech/pharma and wider society will also benefit because of the focus on the "3Rs" agenda - reduction, refinement and replacement of animals in research. This reflects the particular approach that we have taken to modelling cancer which has a major impact on the Reduction of the number of animals used - estimated at 80-90% reduction in our laboratory; and Refinement in terms of reducing animal exposure to noxious toxins to obtain a cancer phenotype (Timeline 0-5yrs). The reduction in animal use will reduce the economic expenditure required to generate new information about cancer.

How will you look to maximise the outputs of this work?

We have a number of strategies to maximise the outputs. First as well as disseminating information through usual scientific channels - publications and conference presentations - we shall use social media and seek to build on my engagement with the public via the NC3Rs Pint of Science event.

Second, our ultimate aim is to improve outcomes in lung cancer and this will require engagement with the biotech/pharma sector. We have multiple existing collaborations with academic and pharma colleagues and will use the data generated to build on these relationships to facilitate novel therapeutic approaches to lung cancer, with the ultimate goal of using our preclinical data to design novel regimes for a clinical trial.

Third, we are developing a suite of genetically modified lines to generate cancers in mice after orthotopic transplantation. For genetic combinations or strategies that are not effective we will also disseminate this information so that the community is aware.

Species and numbers of animals expected to be used

- Mice: 3443

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 main mammalian animal used to model cancer and test potential therapeutic strategies. We can build on the local and global experience of using this model to ensure that 3Rs values are adhered to and we gain the most scientific information from the fewest animals. Our model tumours to date have only been generated in mice. To conclude, no other species is suitable for the type of work that we do.

We will use adult mice in experimental protocols.

Typically, what will be done to an animal used in your project?

Animals for use in this project will be bred or purchased from licensed providers.

A typical experiment would involve the injection of cells under the skin, into the tail vein or directly into the lung under brief anaesthesia, typically isoflurane, an inhaled general anaesthetic. The purpose is to create tumours and the duration of an experiment is typically less than 6 months. Experiments in which a therapeutic agent is administered may typically last an additional 4-6 weeks.

Anaesthesia for tumour induction is typically for a duration of less than 15 minutes. Animals may experience pain at an injection site but this is expected to be mild and transient. The injection of cells directly into the lung, either directly through the chest wall percutaneously or after exposing the ribs using a small incision, or injection directly into the airway, is typically very well tolerated under anaesthesia with no overt signs of pain or distress on recovery from anaesthesia. Cells are administered in a small volume of fluid and using a very narrow gauge needle like a diabetic patient may use to administer insulin. The administration of cells under the skin and into the tail vein has been performed in many laboratories for years and is both well established and well tolerated by the animals.

Animals may or may not be given modified diet or water to activate genes of interest. These are well tolerated in our experience and are not expected to cause distress. Some diets occasionally result in weight loss due to unpalatability. Mashing of diet and supplements such as Nesquik and earlier than usual refreshment of diet will be used to improve palatability. Animals will be placed onto normal diet should they lose 15% of their body weight.

Animals may be anaesthetised for imaging, again typically using isoflurane, during the course of an experiment - typically on two-three occasions. We anticipate a small proportion of animals will undergo imaging (<5%).

Animals will be administered with therapeutics using standard routes (into a superficial vein, under the skin, into the abdominal cavity, via the oral route) and also by direct inoculation into tumours under imaging guidance under anaesthesia. Animals should experience mild, transient pain and no lasting harm from administration of substances by injection using standard routes. The administration of therapeutics directly into lung tumours under anaesthesia is expected to be well tolerated as systemic (body-wide) side-effects should be avoided. Animals will be monitored for signs of lung toxicity.

We will also undertake the administration of therapeutics directly into lung tumours under anaesthesia. This is expected to be well tolerated as systemic (body-wide) side-effects should be avoided. Animals will be monitored for signs of lung toxicity.

Final procedures such as tumour harvest 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.

What are the expected impacts and/or adverse effects for the animals during your project?

Breeding is a natural event with no adverse effect.

Animals could experience mild, transient pain and no lasting harm from the administration/transplantation of cells under anaesthesia.

Animal could experience mild distress only if imaging is undertaken under anaesthesia.

Animals could experience mild, transient discomfort and in most cases no lasting harm from the use of therapeutics. It is possible that a very small proportion (<1%) of animals will have adverse effects due to the administration of therapeutics. This is difficult to predict but animals will be monitored daily for any adverse signs.

Animals that develop tumours may develop signs of adverse effects and for that reason will be monitored daily. Due to the nature of the protocols it is anticipated that mice will not typically experience breathlessness (<1%) but may exhibit general signs of distress with growing tumours. It is anticipated that at most 20% of mice will develop general symptoms as many of our experiments will include animals in which only one lung is affected.

Animals may experience weight loss of up to 15% as a result of change of diet or development of tumours. Attempts will be made to mitigate this if it is caused by diet.

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)?

Breeding and maintenance of wild type mice: 100% sub-threshold Breeding and maintenance of GA mice: 10% mild; 90% sub-threshold Experimental 80% mild; 20% 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?

Cancer is a complex disease that relies on the interplay between the tumour cells and the microenvironment, often made up of cells from the individual's immune system and other cell types. It is not possible to achieve this complexity in vitro nor to understand the impact

of therapeutics that may rely on the microenvironment or cells other than cancer cells for their activity. Therefore, we need to use animals in experiments.

Which non-animal alternatives did you consider for use in this project?

I am committed to the 3Rs agenda. We considered patient-derived organoids and a specific “organotypic” model of early lung cancer that we have developed using immortalised lung cells and genetic changes, and cell lines for this project.

Why were they not suitable?

None of these were suitable because they cannot create the complex in vivo conditions in which a tumour develops or in which therapeutics, particularly those that target the tumour microenvironment, may act. We are using the organotypic model to inform which genetic combinations to use and this has the impact of reducing the number of animals used.

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 and will continue to apply appropriate experimental design methodology including expert statistical support and the NC3Rs experimental design assistant to support experimental design including the randomised allocation of animals to groups and ensuring equal representation of male and female sex animals.

We have estimated numbers based on our prior experience with breeding wild type and genetically altered animals and developing the lung tumour and xenograft model on which this application is based.

Many of the protocols we are using are novel and have been developed in house by ourselves. We are generating complex genotypes in vitro from cells we have taken from the lungs of mice and then genetically manipulated to create multiple molecular combinations of interest. This avoids multiple lines of breeding of genetically altered animals, and when these cells are injected into a recipient and inbred (or syngeneic) mouse it means that the recipient mouse retains an intact immune system. The natural history of specific genotypes and other technical aspects of the project are being refined. Therefore, aspects of this work can be considered pilot work to refine the experimental design, whereas other aspects will use standard experimental design based on our prior experience and the pilot work.

We will use pilot experiments to estimate penetrance and tumour natural history. We anticipate fewer pilot experiments being required with later tested genotypes. Typically, we will use 8-12 mice per genotyped cell line. These mice will be injected with the syngeneic manipulated cells to assess whether the tumours "take" and determine their natural history. Further manipulation will be through the presence or absence of gene activation through adding specific agents to the diet. In certain situations, and for particular genotypes, this may necessitate prior pilot experiments in which the cells are first passaged in an immunodeficient animal by injecting the cells under the skin in a mouse’s flank and allowing them to grow before harvesting them and growing them again in the

laboratory. This has been shown to improve subsequent tumour “take” while retaining key characteristics.

Further dose-finding experiments will be used to ensure tolerability of therapeutics and therapeutic combinations.

In total we estimate 2-400 mice for pilot experiments.

In terms of primary objectives and endpoints a key primary objective for the syngeneic transplantation model is to define the genotypes of cells that efficiently form thoracic tumours after transplantation. The endpoint is proportion of mice developing tumours after 12 weeks. The experiments will be performed using experimental cohorts of about 15 mice, and will be used in an adaptive two-stage design (Simon's minimax two-stage design) performed with first imaging to measure tumourigenesis performed at a predefined interval (anticipated to be 12 weeks but to be informed by the pilot experiments). The experiment will then be terminated with organ harvest at a distant timepoint anticipated to be 24 weeks, after the outcome of the adaptive second cohort of mice is collected. The purpose of the two stage approach will be to have sufficient the power to reject a genotype as being able to deliver thoracic tumours without necessarily having to test a complete cohort – hence an adaptive design and a reduction in overall animal numbers. There will be multiple tested genotype/gene activation combinations so an estimate of 360 (24x15) mice required. Allowing 10% for unforeseen experimental eventualities we would anticipate 396 mice being required.

As per NC3Rs guidance the pilot work may constitute experimental work, particularly in later tested genotypes when the natural history is clearer.

We expect that therapeutic studies will be performed on at least 8 therapeutic approaches. In these experiments the objective is to demonstrate antitumour activity of a target compound on the tumour and the primary endpoint will be tumour volume measured on a tumour growth curve at repeated timepoints.

For these experiments we would anticipate about 24 mice per experiment at two doses in an unbalanced (e.g. 2:1:1) cohort design with an excess of control arm to reduce the overall number of animals required . For 8 compounds this would be 192 mice with a further 10% to cover experimental eventualities so a total of 211.

As noted above, there are data to suggest that tumour grafting success is enhanced after cell passage in vivo in immunodeficient animals. Therefore, cells from a strain such as FVB/N would be genetically manipulated ex vivo and injected for cell propagation and expansion as subcutaneous allografts. Tumours derived in this fashion would be harvested and propagated and re-injected into a syngeneic (in this case FVB/N) animal. We estimate up to 200 animals will be required for this purpose.

As the above experiments refer only to ex-vivo murine cells we will also perform confirmatory more traditional experiments using human cell line xenografts and immunodeficient animals. These experiments will be required by reviewers of our work to confirm the relevance to human disease. For these experiments we would anticipate about 24 mice per experiment at two doses in an unbalanced (e.g. 2:1:1) cohort design with an excess of control arm to reduce the overall number of animals required. For 10 compounds this would be 240 mice plus 10% for experimental eventualities so a total of 264.

Finally, key models for certain types of lung cancer are carcinogen-induced, in particular NTCU (Nnitroso-tris-chloroethylurea). These will be used as orthogonal validation models

for therapeutic experiments and to understand the molecular mechanisms involved in carcinogenesis. We estimate 384 mice used for this purpose. Again we would allow 10% for experimental eventualities leading to a total of 422.

We would estimate an extra 300-400 animals may be required for experimental combinations that are suggested by results so that we estimate 2243 animals in total for the above.

Genetically altered animals. In certain situations, depending on the genotype sought it will also be more efficient (Reduction) to use genetically altered mice (GAA) with specific genotypes. Hence we will have a cohort of GAA mice that will have conditional or germline genetic lesions that are commonly implicated in thoracic cancer/lung cancer.

It may also be appropriate to use inbred lines with specific genetically altered variants to generate the cell lines for subsequent injection into a syngeneic animal. An example may be an oncogene in which the expression level has been optimised and characterised in a mouse mode rather than crude lentiviral transductionl. This will involve purchase or transfer of colonies and their maintenance and breeding for variable periods depending on the allele and background.

Overall GAA estimate to include breeding, colony management, pilot and experimental work is for up to a further 1200 animals over 5 years.

This equates to a total estimate of 3400 mice.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

This work is following on from a project grant in which we demonstrated the potential to reduce the number of animals used in a research project by 90%. Therefore, reduction in animal numbers used is a core aspect of this proposal.

We are careful to use the NC3Rs experimental design assistant (EDA) and the ARRIVE guidelines to guide our approach to animal experimental design.

We employed the (EDA) to plan our experimental design, practical steps and check our statistical analysis utilising the advice and support for randomisation and blinding, sample size calculations and appropriate statistical analysis methods. We can use the EDA outputs to support experimental planning with animal users.

The engagement of a specialist preclinical statistical advisor has been instrumental in innovations which will help reduce the number of animals required. A first example is the two stage adaptive design allowing the discounting of genotypes as being efficient for thoracic tumour formation. A second example is a multi-arm antitumour activity design with a common control group, two active doses and unequal allocation ratio with an excess of animals in the control arm to reduce the overall number of animals (Bate et al, (2014); PMID: 25504147).

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 prioritise genetic combinations based on the literature and our parallel in vitro models. When appropriate, animal tissue will be stored frozen or fixed if analysis cannot be undertaken promptly, so that duplicate experiments are not needed in the future to generate the same material.

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 mainly transplantable models of cancer in mice. The models proposed do not involve any major procedures that are likely to cause more than transient pain or distress to the animals. Many are undertaken under anaesthesia and there has typically been no requirement for analgesia using the methods we describe.

A majority of the models involve cell transplantation through injection into the lung. When only one lung is affected the risk of respiratory/breathing distress is much reduced.

There is a potential for distress due to tumour burden but all experimental mice will be monitored daily to ensure any distress is limited and such animals will be killed. We do no not expect any protocols classified as severe.

Why can’t you use animals that are less sentient?

We seek to model human cancer. The mouse is the best mammalian model system to achieve this because of the global experience in so doing and because of the need for animals with an intact immune system similar to the human one.

This is not a disease of the embryonic stage and therefore adult animals will be used.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be routinely monitored for adverse effects such as changes in weight, piloerection, changes in mobility, lumps, abnormal respiration, or stools. If these are observed animals will be

treated accordingly, and animals that develop significant/sustained effects will be humanely killed according to the protocols in this application.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I am aware of and have read and consulted the PREPARE and ARRIVE guidelines. I also use the NC3Rs website is a valuable resource for video and other communications addressing best practice in animal experiments.

The Laboratory Animal Science Association (https://www.lasa.co.uk/current_publications/) and Research Animal Training websites (https://researchanimaltraining.com/) have practical recommendations and guidelines that cover broad aspects of using animals in scientific experiments, including the administration of substances.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I have regular correspondence with the NC3Rs head office and I receive newsletter email alerts for updates. Indeed, we have recently been the subject of one of their 'impact' publicity social media posts. In addition, I actively check their website for developments. I will continue to engage with the local 3Rs infrastructure which is a priority for my institution.

84. Striatal circuits for learning, memory, and reward processing

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Rodents, Learning, Memory, Reward, single cell recording

Animal types	Life stages
Rats	Adult
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?

A region called the striatum is a central part of the reward circuitry of the brain. By reward circuitry we mean the connected brain areas that identify and learn about things that are rewarding, as well as control behaviour towards these rewards. The first aim of this research is to better understand the role that the striatum plays in reward learning and behaviour. In particular, the striatum is made up of separate parts, called subregions, that are thought to be involved in different aspects of reward processing. For example, one subregion has been linked to determining the value of a reward, in other words, how nice it is, whereas other regions have been linked more to learning about rewards and the things that predict getting rewards. The second aim of this project is to better understand how changes to these brain areas and the ways in which rewards are valued and learned about, change in mental health conditions such as depression and schizophrenia.

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 is important as it will improve our understanding of 2 areas of research. Firstly, it will allow us to learn more about the ways in which rewards are learned about and valued - the 'psychological' basis of reward. Secondly, it will improve our understanding of the roles that brain areas of interest play in learning about, valuing, and driving behaviour towards rewards and things that predict these rewards - the 'neural' basis of reward learning and behaviour. In particular, this work will result in a better understanding of a brain region that is heavily linked to reward value and behaviour, in human and animal studies, the striatum.

This work is also important as it aims to better understand psychological and neural changes seen in mental health disorders. For example, reductions in reward value called 'anhedonia', is a core symptom of depression and many other disorders. Anhedonia has been linked to reduced activity of brain cells in the striatum, the main region of interest to this project. Reduced motivation to work for rewards has also been linked to addiction, depression and other disorders, and has been linked to changes in brain cell activity in the striatum. By better understanding the role that the striatum and it's separate subregions have in learning about and valuing reward, we can create core knowledge about how reward works in the brain. This can then be used to examine how activity of brain cells here, and behaviour towards reward, change during mental health disorders, including depression, addiction, and schizophrenia.

What outputs do you think you will see at the end of this project?

The primary output will be a better understanding of the roles that subregions of the striatum have in learning about rewards, valuing rewards, and motivating behaviour towards rewards. The results will be published open-access where possible, meaning anyone can access them, and also presented at relevant conferences and events. The potential findings from this research are important as it aims to improve core understanding of how the brain learns about and determines behaviour towards rewarding things. This will allow future research to look even more at the changes in reward learning, value, and behaviour that occur in mental health disorders such as depression, and ultimately develop more effective treatments for them.

Another output are the data sets generated, that will include very large data sets of the activity of many brain cells (neurons) in brain regions of interest. This data will also be made freely available to other researchers, allowing it to be used to look at important research questions about the brain and reward, without using additional animals.

Finally, it is important that researchers look at new ways to reduce animal numbers. The next generation recording techniques used in this project are still new, so projects like this one will help to develop best practice alongside increasing quality and quantity of data collected from each animal.

Who or what will benefit from these outputs, and how?

In the short-term, benefits are to researchers studying how the brain learns about, values, and determines behaviour towards rewarding things and things that predict reward. Especially in relation to the core region of interest, the striatum, and its known subregions. It will also indirectly benefit researchers by providing new data sets for further use by the research community. Additionally, a large proportion of research, in humans and animals, uses reward-based procedures to study the mind and brain. Therefore, better understanding of these circuits can inform refinements to, and directions for, future experiments.

In the long-term, this work could provide valuable insights into the how changes in the mind and brain underly mental health disorders such as depression, addiction, and schizophrenia. All of which are associated with changes in learning about, valuing, and behaving around rewarding things. This would benefit clinicians and researchers looking at diseases and disorders such as anxiety and depression.

How will you look to maximise the outputs of this work?

Wherever possible the findings from this research will be published Open Access in the best journals with wide audiences. The data will also, wherever possible, be made available through free to access databases. This would allow other researchers to make use of this dataset and avoid the need for similar animal studies. The work will also be presented at national and where possible international conferences, to present the work to as many other researchers and research groups as possible. Another method of output is the potential for collaboration with other research groups, that would expand the impact and potential of this research.

Species and numbers of animals expected to be used

- Mice: 1000
- 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.

Rodents will be used as they have brain structures sufficiently similar to the human brain, including the key region of interest to this project, the striatum. They are also the species that the particular equipment being used in this project to measure the activity of brain cells has primarily been developed for. There are therefore many resources and the knowledge available to ensure high quality data while maximising animal welfare. Finally, there are decades of previous research in rodents understanding their brain and behaviour that this research will be building upon and allows for suitable experiments to be carried out.

Adult rodents will be used to record the activity of brain cells from fully developed adult brains. They are also better for animal welfare, being fully grown and most able to undergo the behavioural tasks and surgery required for this project.

Rats and mice will both be used. Mice will be the first choice and used in the majority of studies. This is because of the existing research in mice, with the brain cell recording technology necessary for this project. There is also the benefit of the smaller brain size, meaning that with existing technology brain cell activity can be recorded from across more areas of interest to the project compared to rats.

However, in some cases rats provide the better model of choice. One reason for this is their larger size and weight making them more suited to undergoing the surgery necessary to insert the small recording device into the brain areas of interest to this project. They may therefore be the best choice for animal welfare when longer testing periods are necessary to achieve the scientific aims of the project. There are also some behavioural tasks better suited to rats over mice due to differences in species behaviour e.g., greater eagerness to

participate in tasks, superior maze learning, and faster ability to learn about reward predicting cues. Rats may therefore also be beneficial when the behavioural task requires a shorter training and/or test period, as well as when longer periods of stable behavioural responding are required.

Typically, what will be done to an animal used in your project?

A typical animal will undergo approximately 3 months of testing centered on behavioural training using mild food restriction to motivate behaviour. For most experiments this will involve learning about auditory and visual cues leading to food reward, and/or training to make certain behavioural responses to earn food reward. This would generally take place in a behavioural testing chamber for mice or rats, with daily testing sessions lasting no longer than an hour and generally for 5 days a week. They will also be group housed during this stage as far as possible and provided with enrichment in the home cages. For a minority of experiments rather than learning about cues in test chamber, they may instead be trained and tested on a maze task to test spatial learning. These could include dry or water mazes, in which animals learn to go to a correct location or make a certain pattern of movement to earn a reward. Maze testing, including the Morris Water Maze, would be carried out in line with standard testing procedures. For water maze testing this involves a training phase where the animal learns the location of a platform in the maze and to swim to it, followed by a short test phase where animals are tested with the platform removed to see if they search the correct location the platform was previously located. During these test sessions they are removed from the water maze as soon as the trial duration is reached, with this duration the same as the maximum trial duration used during training. Some animals will remain in this behavioural stage, undergoing various behavioural tests to further understand the way in which the mind learns about reward, values reward, and motivates behaviour towards reward of different types.

For the rest of the animals (up to 70% of animals), the next stage would be surgery under anaesthesia. This would be carried out after ~4-6 weeks of behavioural training, or the time taken to establish the behaviour of interest. Animals would not be on food restriction when surgery takes place, and surgery would typically last around 2-5 hours. During this surgery, animals would experience one of two similar paths, and never more than one of these.

Pathway One

A small recording device will be inserted into the brain region of interest, and necessary components, such as the connector, permanently attached to the skull. Animals will be appropriately monitored and cared for before, during, and after surgery e.g., body weight, body scores, and painkiller administration. The mice or rat(s) would be in a recovery period for around 7-10 days but can be extended so that only after full recovery would animals be continued to the next stage of the study. The total duration of this surgery and recovery stage is ~2-3 weeks. The final stage is then the recording stage, lasting 2-4 weeks, when activity of brain cells are recorded during the behavioural task. In this stage the mouse or rat is returned to the same behavioural task they were trained in before surgery, under mild food restriction to motivate behaviour. Daily recording sessions will typically last 30 – 45 minutes, up to an hour maximum and generally carried out up to 5 days a week. During each session, the mouse or rat will first be lightly restrained in a towel, something they readily accept with only minimal training, while the lightweight recording cable is connected to the connector attached to the skull. They are then placed into the testing chamber, or in some cases the open maze, and neural activity recorded while the behavioural procedure is completed by the animal. Animals and the corresponding neural

activity will be closely monitored throughout the recording session. After this stage is complete, animals will be humanely killed.

Pathway Two

Surgery will be carried out that allows the activity of brain cells to be changed. Examples of this include injecting substances that destroy groups of cells creating a 'lesion', or substances that allow, at a future time, the brain cells to be turned on or off with light or specific drugs. After the recovery period of 7-10 days, animals will be returned to the behavioural task to assess behavioural changes resulting from the changes in brain cell activity. Behavioural testing would be the same as pathway one, with daily sessions usually around 30 – 45 minutes, up to an hour maximum and generally carried out up to 5 days a week. For some studies this may involve further substance administration, typically by injection, to cause the brain cell activity to be changed during the behavioural task. This stage would generally last 2-4 weeks and once complete the animal would be humanely killed.

For some animals on this second path, during the same surgery described above, a recording device may also be inserted into the brain. For these animals there would be the addition of recording of brain cell activity during behavioural testing, in the same way as animals on pathway one.

What are the expected impacts and/or adverse effects for the animals during your project?

Appetitively (food) motivated behaviours (>80% of all tasks)

Food restriction

Weight loss of up to 15% from the animal's starting weight for the duration of the experiment, and temporary weight loss of up to 20% before daily feeding takes place. To monitor this, animals will be weighed before and during the period of food restriction. If an animal drops below 85% of its starting weight, it would be given extra food or returned to free feeding and closely monitored until it has returned to its target weight. Short-term body weight would not be expected to fall below 80% of starting body weight. If any animal on food restriction becomes sick it will be immediately returned to free feeding.

Aversively motivated behaviours (<20% of all tasks)

Conditioned taste aversion

This involves injection of lithium chloride to induce feelings of sickness in the animal. Symptoms are observable through behaviours including hunched posture and isolation from cage mates. These symptoms are expected to improve within 12hrs and not last longer than 24hrs. If an animal shows any signs of sickness beyond 24hrs, frequency of animal monitoring would be increased over the next 24hr period and behavioural testing would not begin again until complete recovery demonstrated by normal home cage behaviours.

Fear conditioning

A very brief mild electric shock is used to generate conditioned fear behaviour leading to brief discomfort, similar to that experienced during a brief static shock. To avoid excess discomfort, the shock will typically be set to 0.5mA for rats, and 0.3mA for mice, never exceeding 1mA for rats or 0.5mA for mice. It will also be limited to a 1s duration and

typically be 0.5s in duration. These parameters are similar to the current experienced during a brief static shock.

Surgery

Post-surgery distress (<1% of cases). Animals may show distress observed with behaviours such as hypoactivity, and excessively hunched postures. If this situation persists for more than 5 days, advice would be sought from the NVS and other researchers, and if recommended the animal will be humanely killed.

Infection (< 2% of cases). The wound may become infected and cause discomfort. The NVS will be consulted and, if appropriate antibiotics will be given. If wound does not heal appropriately the animal will be humanely killed.

Headcap damage (< 15% of cases). The external components of the device used to record brain cell activity, that are fixed to the skull, are called the 'headcap'. This headcap may become loose or damaged. In this case the animal will be briefly re-anaesthetised and the headcap repaired and/or resecured in place one time only. If this cannot be successfully accomplished the animal will be humanely killed.

Headcap loss (< 10% of cases). In rare instances, it is possible that the headcap may come off the skull. In this case, the animal will be immediately and humanely killed. In consultation with advice from the NVS and other researchers, we are working to minimise this happening at both the surgical stage (e.g. procedures used to fix the attachments to the skull,) and afterwards (e.g. modifying cages and the connection between the headcap and recording cable).

Single housing: after surgery to assist recovery and protect the implanted headcap animals will be single housed. This has the potential for adverse effects of isolation such as increased anxiety. This is more of a concern for rats that are more of a social species than mice. This will be reduced with regular handling and where possible developing cages that still allow the animals to see and smell each other, called 'in proxy housing'.

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)?

Rats

Expected mild (30%); expected moderate (70%).

Mice

Expected mild (30%); expected moderate (70%).

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?

Achieving the scientific aims of this project requires the use of conscious freely moving animals. This is because the main method of recording brain cell activity used in this project requires implanting recording devices into the brain during surgery. Methods that exist to measure brain cell activity in humans are unable to record cell activity at the level of detail necessary for the regions of interest to this project. Instead, they are only able to record large areas of the brain and the overall activity of the millions of brain cells within those areas.

Additionally, using freely behaving animals, rather than brain tissue or cells, is required as the aims of this project focus on better understanding the mind and brain mechanisms of reward learning, value, and liking. This therefore requires animals that can complete reward based learning tasks and behaviour. This includes the water maze testing procedure, in which we are testing how well animals are able to learn and navigate towards the location of a hidden platform. As the testing procedures require freely behaving animals, non-animal alternatives are limited, including for the water maze testing procedure.

Which non-animal alternatives did you consider for use in this project?

For behavioural testing, including water maze testing, the requirement for freely moving animal behaviour means that non-animal alternatives are highly limited to achieve the scientific aims of the project. However, there are a couple of alternatives that have been considered.

- Cell and/or brain tissue samples (in-vitro) designed to study learning and memory mechanisms at the level of the brain cell(s).

- Computing technology that models the mind and brain using information already known about them, to predict and test novel research questions.

Why were they not suitable?

Computer models are extraordinarily simple compared to the complexity of the human brain. They are therefore unable to be used to answer the scientific aims of the project of better understanding the mind and brain underlying reward learning, value, and behaviour. This is the case for all the behavioural testing used in this licence, including water maze testing.

With brain tissue and/or cell samples we are unable to study the behavioural processes of interest to this project, of reward learning, value, and other related behaviour. We are also unable to record the activity of brain cells during awake freely moving behaviour.

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 amount required to achieve the scientific aims of the project, with consideration of how many can be tested over a 5-year period.

Breakdown of animal use per species.

Mice

Typically tested in groups of 16-32 animals, with, funding dependent, a group undergoing testing every 2-3 months. For example, in one year there may be 4 smaller groups of mice (~16 animals) and 2 larger groups (~32 animals) that would lead to ~320 animals used in that year. The larger total number (1000) is to include the additional numbers required for breeding and maintenance of genetically modified animals. Many strains require breeding more animals than necessary to reach the required number of the desired genotype, as well as maintain the line over time, for example but not limited to,

GluA1$^{-/-}$ mice. In this case strategies are used to maximise animal use, e.g., using animals as littermate controls. Rats

Typically tested in groups of 8-16 animals, with, funding dependent, 2-4 groups per year.

Water maze testing would typically be carried out using rats, with group sizes of 20-30. This is the minimum number that allows for counterbalancing of key factors and inclusion of experimental and control groups, and is also in line with previous research using the water maze. The number of water maze studies that will be carried out is expected to be 1-4 over the duration of the licence, depending on the progression of the scientific objectives and funding.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Multiple steps will be taken to minimise the number of animals used to meet the scientific aims of the project. Statistical analysis will be carried out to calculate the minimum number of animals necessary, including the use of a power calculator. The NC3R's experimental design tool will also be used during the experimental design process. During behavioural studies within-subjects designs, that changes experimental variables within animals rather than between animals in separate groups, will be used as far as possible. This avoids the use of multiple groups of animals comparing key factors of interest. During experimental design other factors that might affect the results, but are not of primary interest, will be taken into account and controlled for. This allows other explanations of the data to be ruled out and reduces the need for replications and designs with multiple groups.

In studies involving surgery and recording of brain cell activity, the number of animals will be minimised by using recording technology that records many cells, across many brain regions, within each animal. This avoids the use of multiple groups of animals to record across brain regions of interest.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Animals not undergoing surgery will typically be used in multiple behavioural tasks. This will allow greater insight into behaviour and reward processes of interest, such as reward learning and value, without the need for multiple groups of animals.

For animals undergoing surgery and behavioural testing, behavioural training will generally be carried out to ensure each animal shows the behaviour of interest before surgery and recording of brain cell activity. This means that the data collected will be of high quality and able to be used to support the scientific aims of the study. This avoids the need for larger surgery groups, and maximises the the usefulness of the data collected from these animals.

Finally, all lab workers will be trained to a high standard to minimise any errors during surgery, handling, and brain cell recording procedures. This will maximise the quantity and quality of data collected from each animal, and again prevent the need for more animals than necessary.

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 because of the extensive knowledge of their brain structures and biology necessary for the scientific objectives of this research. Appetitively (reward) motivated behaviour that rodents readily engage with will be used in most studies. This allows us to study learning and memory processes with the least amount of stress to the animals. Only when specifically required to meet the scientific objectives will aversively motivated behavioural procedures be used. Brain cell recording technology optimised for use in rodents will be used as these allow large numbers of brain cells to be recorded from within each animal while minimising pain and suffering to the animal.

Water maze testing will be used when required to investigate spatial learning and memory mechanisms specifically. The water maze procedure allows to look at how animals can learn about spatial locations and navigate towards them, something that the other behavioural procedures in this licence are unable to purely test. This is important given that many of the brain regions and neural mechanisms of interest to the scientific aims of this licence have been linked to spatial learning, and water maze testing provides a way to investigate if these regions are linked to spatial learning specifically or more domain general learning and memory mechanisms.

Why can’t you use animals that are less sentient?

Rodents will be used for multiple reasons.

Their brain structures have clear overlap with the human brain, including the brain structure of key interest to this project, the striatum.

Studying the mind and behavioural processes of interest to this research project requires the use of conscious freely behaving animals such as rodents. The tasks used in this research to measure reward behaviour, learning, and other related processes, have also been designed for rodents.

Genetically modified rodents provide a unique way of assessing the underlying causes and changes to the mind and brain that occur in mental health disorders. For example, animals with altered genetics can be used to test the role of genes that have been linked to mental health disorders and their symptoms in humans.

Lower sentient species or invertebrates cannot be used given the scientific objectives of the project.

This is because to investigate the psychological process of interest, including reward learning, memory and related behaviour, requires freely behaving animals. In addition, the brain regions of interest to this project, the striatum and connected structures, do not have the same overlap in these species.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Researchers will be extensively trained in animal handling, surgical techniques, brain cell recording, and animal monitoring procedures to minimise stress to the animals and maximise animal welfare. During behavioural testing, animal behaviour and task progress are closely monitored so that it does not extend beyond what is required to achieve the scientific aims of the project.

All surgery will be carried out under clean (aseptic) procedures as far as possible. Animals will also be extensively monitored before, during, and after surgery, including giving pain relief before and after until fully recovered. After surgery, when animals are required to be housed alone, we aim to use housing being developed that allows animals to interact (see and smell) each other through mesh partitions of larger housing.

Where possible, other refinements will be considered, such as housing and testing under reverse light cycles so animals are tested during their natural active period.

For water maze testing the training and test procedure has been refined to maximise animal welfare. This includes having a water temperate that is optimal for preventing distress or discomfort, trial durations are kept short while allowing sufficient data to be collected, and animals are immediately dried of after each trial to prevent animals getting cold while wet.

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

As the technique for brain cell recording is highly specialised, refinements for these will be gained through keeping up to date with related literature, the scientific community, and

through contact with other researchers using similar techniques. This will ensure that refinements are known about and can be implemented as soon as possible.

Water maze testing will be carried out in line with established refined testing procedures, including optimal water temperatures, trial durations, and drying times.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed through multiple means. We will keep up to date with literature relevant to the 3R's, assisted by mailing lists such as PubCrawler to be notified regularly of newly available literature. We will also attend conferences, NC3R's seminars & events, and engage with material on the NC3R's website. 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. Finally, we will regularly review procedures to refine and use relevant advances that have been made wherever possible.

85. Novel ligands for cell type-specific drug delivery

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

Drug delivery, Cancer, Targeted therapy

Animal types	Life stages
Mice	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 identify new ligands that, when injected into animals, will accumulate at specific cell types and organs, with the view that in the future the ligands will be coupled to therapeutics for targeted drug delivery in 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?

Targeted drug delivery is a major unsolved problem in biomedicine. Most drugs, when injected into a patient, will distribute non-specifically throughput the body, accumulating in the liver or filtered out by the kidneys. For many diseases, this results in a subtherapeutic (ie. ineffective) local drug concentration at the organ of interest, as well as toxic off-target effects due to drug accumulation in non-target organs (eg. liver toxicity toxicity). To

address this problem, we must identify novel binders that display cell or organ-specific binding and accumulation. With these tools, we will be able to better treat diseases such as cancer (eg. targeted delivery of chemotherapies).

What outputs do you think you will see at the end of this project?

The main outputs to this project will be ligands that enable drug delivery to specific organs or cell types, with improved efficiency and specificity compared to existing modalities. We envision that our ligands will be applicable to deliver a wide range of different drugs, including chemotherapy for use in cancer therapy, as well as different classes of genetic medicines. The details of these experiments will result in peer-reviewed publications and patents. We will also present our results in local and international conferences.

Who or what will benefit from these outputs, and how?

The broader academic scientific community will immediately benefit when we disseminate our results (within 3 years). On a longer time scale (5-10 years), we aim to translate results obtained in our animal studies into novel drug candidates that will begin clinical trials; this will directly benefit patients by increasing patient survival through increased drug efficacy, or by decreasing side effects of existing drugs through reduced drug accumulation in off-target tissues.

How will you look to maximise the outputs of this work?

We aim to publish our findings in peer-reviewed journals, as well as present our results in local and international conferences, both within academic and industrial settings. We also aim to maximise our outputs by working with different academic groups and allowing dissemination of our findings through collaborations.

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 are broadly interested in targeted drug delivery to a wide range of organs, in individuals that are healthy as well as tumour-bearing. Consequently, we will use wild-type mice, as well as a subcutaneous (where tumour cells are introduced under the skin) tumour mouse model. We will use mouse models because cell lines, patient samples, and other complex cell culturing techniques (eg. organoids or organ-on-chip) do not accurately recapitulate the complex interactions between different organs in a live animal, as well as certain properties in the blood of live animals which affect how quickly drugs are degraded when injected, that are particularly important when characterising drug biodistribution and half-life.

We will use juvenile, adult, and aged mice to test drug delivery candidates. This will allow us to assess whether the age of an individual affects the efficacy of our delivery candidates.

Typically, what will be done to an animal used in your project?

Our experiments will involve both healthy and subcutaneous tumour-carrying mice. The former will simply be obtained through breeding of "wild-type" mouse colonies. For the latter, our experiments will begin with tumour induction. This is achieved by injecting animals with tumour cells. Tumour development will be assessed by calliper measurements (tumour size), imaging, and blood sampling.

Once healthy or tumour-carrying mice are obtained, delivery modality candidates (sometimes linked to relevant drugs to test their delivery) will be administered into the bloodstream. At a defined time point (generally after several hours, but could also be across several days), animals will either be imaged (for appropriately labelled candidates) to assess the biodistribution of the injected drug, or they will be killed humanely to allow biodistribution across multiple organs to be assessed by extraction from organs. Regardless of experimental workflow, all animals will be killed humanely at the end of each experiment.

What are the expected impacts and/or adverse effects for the animals during your project?

General signs of distress or ill health will be carefully assessed throughout each experiment.

Tumour induction may cause mild pain and discomfort, though we have found that tumour growth is generally well tolerated. Potential adverse effects include weight loss, hunching, tremors and altered breathing, as well as general behavioural patterns associated with distress such as excessive grooming. However, these are very rare. We will closely monitor tumour-bearing animals, and any mouse displaying any such signs of discomfort will be humanely killed.

Administration of our drug candidates may cause very temporary discomfort associated with any injection. Beyond these effects at the site of injection, from our experience we do not anticipate any other side effects caused by our drug candidates. Nonetheless, any animal displaying sustained signs of discomfort after injection 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)?

We plan to use roughly equal numbers of healthy and tumour-bearing mice.

All healthy mice used in our experiments are expected to experience no more than mild suffering, as they will only endure drug / delivery candidate administration through injection into the bloodstream, whole body imaging to assess biodistribution of administered candidates, before being killed humanely.

Tumour-bearing mice are expected to experience no more than moderate suffering, due to the injection of tumour cells, as well as some pain and discomfort caused by the resulting tumour mass. Unlikely (<5% animals) adverse effects for tumour growth include weight loss, hunching and tremors, but in our experience we have not come across these effects. Animals experiencing these adverse effects will be killed humanely.

From this, we envision that roughly 50% of animals will experience mild severity, and 50% of 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?

The aim of our project is to accurately characterise the distribution of different candidates for drug delivery when injected into live animals (known as drug biodistribution). While we use other non-animal models in the laboratory to assess distribution and uptake of drugs across different cell types, ultimately these poorly recapitulate key aspects of drug distribution and accumulation inside patients.

This is largely because (1) different organ systems within the body interact with each other in complex ways that can result in unpredictable patterns in drug biodistribution, and (2) properties of the blood within live animals often lead to decreased stability of a drug, which can only be accurately assessed in live animals. Therefore, in order to accurately assess the efficacy of a given candidate for drug delivery, live animals must be used.

Moreover, we have searched through existing publications and pre-prints (and will continue to do so during the course of the project) to ensure that we are not duplicating experiments already performed elsewhere, including the SyRF platform to access meta-analysis of existing animal work.

Which non-animal alternatives did you consider for use in this project?

We have used, and will continue to use, alternatives in assessing the potential of different candidate modalities for targeted drug delivery. These include healthy and tumour-derived human cell lines, cells isolated from healthy patient blood samples, as well as cancer tissues and blood from cancer patients. In order to preliminarily assess biodistribution across different cell types without using live animals, we often co-culture cells from different organs to assess differential uptake of drug candidates across different cell types. We only carry out animal work for a given candidate if we find promising results in prior, non-animal studies.

We have also considered the use of organoid and organ-on-chip systems, as well as non-protected species, but unfortunately these are poor models for biodistribution in humans.

Consequently, we need to use mice because they are well characterised to have physiology that is similar enough to humans to yield meaningful experimental results.

Why were they not suitable?

Biodistribution is a complex, emergent property that arises from the interactions of different organ systems within a living patient or animal. Non animal models, including ones we use (such as human cell lines and patient derived samples), as well as other systems (including organoid and organ-on-chip models) are unable to accurately recapitulate and mimic drug biodistribution in patients. In particular, using live animals, we can accurately assess drug serum half-life, unwanted accumulation in off-target tissues, as well as the effects of using different delivery schedules, as well as the age and sex of the animal. Moreover, non-protected species, including fruit flies or nematodes, are insufficient for our purposes because, unlike mice, their physiology and anatomy are too different from that of humans to be of predictive value. For these reasons, we argue that it is essential for our work to be conducted in 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?

Our estimated number of animals is calculated based on our planned research activity, as well as past studies assessing biodistribution of related drug delivery modalities.

In particular, we plan to inject a large collection (tens to hundreds of thousands) of drug candidates into a single animal, and assess biodistribution of all candidates through post-mortem analysis of organs. Past studies have shown that group sizes of 8-10 animals (biological replicates) are required to achieve statistically significant results in such experiments. In these experiments, control animals are not required, because the pool of injected candidates will contain negative controls which allow for internal standardisation within experiments. Importantly, the drug candidates used are not expected to exhibit any harmful effects to the animals, based on our past experience and on studies from other laboratories in the published literature.

The total number of animals estimated amounts to 800 animals used per year across a 3 year period. This accounts for numerous lines of experiments performed by multiple scientific staff members. We envision a roughly equal split of experiments looking at healthy animals vs tumour-bearing animals. This amounts to 400 animals used in protocol 1, and 400 animals used in tumour induction protocol 2, in each year. For each stream, we envision this to be composed of 10 biodistribution experiments of 10 animals each, followed by roughly 15 validation studies to assess individual hits resulting from these screens requiring 20 mice each (10 with drug candidate, 10 with control).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

A major aspect of our experimental design is our ability to evaluate large (tens to hundreds of thousands) collections of candidates in a single experiment, due to the nature of our drug candidate modality. This is unlike most other animal studies of drug biodistribution, where drug candidates must be tested individually. Because of this, we are able to massively reduce the number of animals used, compared to an analogous experimental design where each candidate is tested in separate animals.

Moreover, we have used the NC3Rs' experimental design guidance and assistant (EDA) to design our experiments in order to identify nuisance variables (eg. age of animals, time of day of drug administration etc), and eliminate associated experimental noise. This will allow us to achieve greater statistical power with fewer animals. By consulting published studies on pooled drug delivery screens employing a similar library size, we conclude that 8-10 mice per experiment will achieve sufficient power in our statistical analyses.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where possible, we will obtain transgenic mouse strains from collaborators or commercial providers instead of producing new strains ourselves. Relatedly, all breeding under this license will be managed by experienced technicians to ensure 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.

Mouse models

Mouse tumour models will be used, because mice are the most well-characterised, and lowest form of mammal that is relevant for modeling human cancers. In our previous work, we found that our most promising drug delivery candidates show specificity to tumour cells from many different organ types when tested *in vitro*, and consequently in this project we will test whether this translates to *in vivo* biodistribution. These tumour mouse models will enable us to determine whether our candidates are capable of specifically delivering drugs to tumour cells, while sparing neighbouring healthy cells and in other organs of the same animal. Moreover, we will perform experiments on animals at a relatively early time point after tumour induction, allowing us to minimise discomfort in animals due to tumour burden. We will carefully characterise tumour burden (eg. using bioluminescence imaging and calliper measurements) in order to perform experiments as early as possible, before animals experience significant pain or suffering. Moreover, we will carefully monitor the health and behaviour of all animals. If necessary, animals will be humanely killed to avoid further suffering.

Methods

Throughout the project, we will use methods that will minimise pain, suffering and distress in all animals. We will administer individual drug candidates or large pools of candidates through tail vein injection, which causes suffering of a mild severity. We will follow best practices to reduce harm to animals (eg. only use single-use needles to avoid pain from using dulled needles). Our work will be carried out by staff members that are fully trained, ensuring that the highest level of animal handling.

Why can’t you use animals that are less sentient?

Mice are the most well validated mammal for cancer models, and are highly relevant in biomedical research due to the similar physiology between mouse and man. Consequently, relevant mouse disease models will be able to recapitulate key aspects of drug biodistribution, enabling accurate characterisation of specific drug delivery candidates. Unfortunately, such experiments are simply not possible in less sentient animals, because their physiology is not similar enough to that of humans. Relatedly, embryos or very young animals are also insufficient for our purposes, because their organ and circulatory systems are not well developed enough to fully recapitulate drug biodistribution patterns in human adults.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Throughout the work described in this license, we will continue keeping up to date with relevant literature and NC3R guidelines to ensure that our procedures are of the highest standards. For chemically induced tumour models, which may involve repeated injections over multiple weeks, we will ensure that where possible, animals are handled by the technician prior to tumour induction, allowing acclimatisation and reduced distress during the experiment. We will also use environmental enrichment to minimise stress. Moreover, we will closely monitor animals for signs of pain and suffering.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will adhere to the NC3Rs ARRIVE guidelines and the PREPARE guidelines, as well as other publised guidelines (eg. https://doi.org/10.1038/sj.bjc.6605642).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We plan to stay informed about advances in the 3Rs by regularly checking available information on the NC3Rs website. We are also subscribed to the NC3Rs newsletter, and we plan to attend regular local 3Rs symposia.

86. Poultry Coinfection Epidemiology, Immunobiology and Vaccinology

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

Poultry, Coinfection, Immunobiology, Vaccinology, Epidemiology

Animal types	Life stages
Meleagris gallopavo domesticus	Neonate, Juvenile, Adult
Domestic fowl (Gallus gallus domesticus)	Embryo and egg, Neonate, Juvenile, Adult
Phasianus colchicus	Neonate, Juvenile, Adult
Alectoris rufa	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?

This programme of work is to gather further information on the complex interactions between poultry (host) and co-infection of viruses and mycoplasmas that cause diseases, welfare concerns, production losses and eggs/meat wastages in the UK.

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 UK currently produces about 1.3 billion broiler and 13 billion table eggs for human consumption. In addition, the UK has a substantial number of pure breeds, great-grand/grand, and parent stock farms.

The proposed research concentrates on poultry infectious diseases (caused by infectious bronchitis virus, avian metapneuvirus, *Mycoplasma gallisepticum* and *Mycoplasma synoviae*) that are endemic in UK, where farmers and vets, facing poultry health, welfare and production issues on a daily basis. These pathogens, either as single or mixed infection (where one promotes the ill effect of other) causes higher mortality, substantial decrease in the body weight gain, increases feed conversation ratio, lower egg production or eggshell quality, and in some severe/complicated cases antibiotics are used to control the problem. The studies proposed in this application will provide provide scientific evidences for better control strategies, for example, early or combined vaccination, optimisation of immune responses through better vaccine-take, identification of appropriate field risk factors in relation to infection process, identification of immune markers for 'immunosuppression', development and efficacy testing of new vaccine or vaccination programmes.

Therefore, it is important to undertake the proposed work, i) to sustain our supply of poultry meat and eggs through better control of infectious diseases, ii) to cut down production wastages (meat and eggs) in farms and abattoirs through improved control of infectious diseases, iii) to enable research and development of new vaccine technologies and vaccination programmes, iv) to sustain and further enhance poultry research in the UK through training of postgraduates and technicians, v) to sustain the global expertise and knowhow of UoL in in-vitro, in-ovo and in-vivo poultry work.

Due to similarities in poultry production worldwide, research outputs will benefit all communities (e.g., scientists, veterinarians, producers, and policymakers) globally engaged with poultry.

Significantly, the proposed work is important in sustaining poultry health and production and improving their welfare.

What outputs do you think you will see at the end of this project?

The proposed research will generate multiple outputs. This included the following:-

Improving poultry diagnostic assays. Through our science-based research activities, this proposed research aims to produce lateral-flow test kits and recombinant ELISA for more efficient detection of M gallisepticum (MG) and M synoviea (MS), mycoplasma of major concerns in poultry globally. Presently, the recombinant proteins of MG and MS have been synthesised, and soon after obtaining this project licence, they will be validated through experimental work on the birds.

Leading innovative vaccine developments against poultry viruses and mycoplasmas. With our thorough understanding of poultry infectious diseases and vaccine usages, we are developing a saRNA vaccine against infectious bronchitis virus (IBV) and recombinant vaccines against (MG) and (MS). For both vaccines, the first part of the activities has been completed. Upon approval of this project licence, the PhD students can proceed with bird experiments. Both innovative vaccines would be highly useful for the control of IBV, MG and MS, respectively.

Impact on global poultry farming. Our research activities are science/evidence-based for enhancing poultry welfare, health and production globally. Our work on live viral vaccine

interactions has been shown to provide protection against 2-3 different viral challenges. Global producers have adopted our vaccination programmes for better poultry health and production. The programme also contributed in reducing disease pressure in flocks and increased farmers' income in developed and developing countries.

Safeguards poultry welfare, health, and production. Reducing infectious bronchitis virus (IBV), avian metapneumovirus (AMPV), Mycoplasma gallisepticum (MG), and Mycoplasma synovial (MS) infections, either in single or mixed cases, leads to better control of infectious diseases in the farm and backyard settings, resulting in better welfare, health, and production.

Reducing poverty and malnutrition in some countries. Reduction of carcass condemnation at slaughterhouses, and reduction in loss of eggs, and mortality in farms due to these pathogens. In certain countries, vaccine and vaccination innovation findings of our studies have resulted in better output for farmers, thus increasing household income.

Who or what will benefit from these outputs, and how?

As one of the leading global poultry respiratory research groups, our outputs are received with high credibility. Our outputs will be continuously shared with major stakeholders in the world and UK industry. This includes the following institutions: -

i. Regulatory bodies (e.g. DEFRA, APHA, European Pharmacopeia, WOAH, Veterinary Medicine Directorate) ii. Academia/research institutions (e.g. Universities, Pirbright Institute) iii. Biological and pharmaceutical companies (e.g. MSD, Boehringer, Zoetis etc).

Veterinary poultry organisations (e.g. British/World Veterinary Poultry Association, British Veterinary

Association)

Poultry producers and their associations (e.g. Aviagen, Cobb, Lohmann etc).vi. Other non-governmental organisations (e.g. National Farmers Union, RSPCA).

vii. The proposed work will contribute to a sustainable supply of antibiotic-free, welfare-friendly eggs and meat to UK and global consumers.

These organisations tend to utilize the data for better poultry welfare, health and production. In some cases, it becomes a means of improving the income of smallholder and backyard poultry farmers in developing countries.

In general, this proposed project intends to provide short- and long-term benefits in poultry disease prevention and control. These are outlined below.

Short-term benefits:

Identification of suitable vaccines for the best immunization in flocks. New strains/genotypes of RNA viruses, such as IBV and aMPV, emerge infrequently due to recombination or point mutations, and we foresee the continuous benefit of providing solutions regarding this to the UK poultry industry.

Optimised protection against IBV, aMPV, MG, and MS through strategic and science-based vaccination programmes. Currently, we are developing saRNA IBV and recombinant protein MG and MS vaccines. It is possible that within the next 3-4 years, the vaccine research could be concluded.

Use of immunomodulators to enhance antigen presentation for better respiratory vaccine uptake and immunity. Research related to this will run alongside the vaccines and may take about 3-4 years.

Development of safe, efficient and affordable live vaccine delivery methods, e.g. natural ‘gel-like’ substances that could sustain better and prolonged viability of live vaccine viruses, and for smoother welfare-friendly administration. With the availability of an increasing number of poultry vaccines, the delivery of multiple vaccines in administration has become a norm in the poultry industry. This study is expected to be conducted throughout the project's 5-year duration.

Better control of respiratory viruses and mycoplasmas would reduce exacerbation by bacterial pathogens, leading to reduced usage of antibiotics. This would promote a reduction in antibiotic usage and avoid AMR. This is basically achievable through full development of the use of MG and/or MS vaccines, likely within 4- 5 years.

Long-term benefits:

Reduction in IBV, AMPV, MG, and MS infections, either in single or mixed, leading to better poultry welfare, health and production.

Reduction of carcass condemnation at slaughterhouses, reduction of loss of eggs and mortality in farms due to these pathogens.

Development of new and innovative vaccines for stress-free administration and effective control of diseases.

Enhanced upper respiratory induction with appropriate vaccines for better poultry respiratory health throughout their lifetime.

How will you look to maximise the outputs of this work?

The output of the proposed research is maximised by adopting the following:-

Innovation. By incorporating many recent laboratory, epidemiological and technologies into our research protocols. This includes cell sorting and mass cytometry, cell bioimaging, transcriptomics and proteomics. Collaboration - Industry (UK, Europe and Rest of the World)

Collaboration. We formed intra-university collaborations to optimise our results, analysis, and global acceptance. Beyond the University, we collaborate with other universities, research institutions, and government agencies.

Other Publications. In addition to high-impact journals, we publish in farmers’ and veterinarians’ magazines and certain Asian journals for regional impact.

Online resources/library. Our genetics findings, such as sequences and amino-acid profiles of new and conventional virulent and avirulent pathogens, are shared freely and are available via Online libraries/resources, e.g. GenBank.

University & collaborators websites. We uploaded much of the unpublished research and technical data via websites of University and other trustworthy partners. This included research protocols, data and scientific research findings.

Species and numbers of animals expected to be used

Domestic fowl (Gallus gallus domesticus):

460 Other birds:

Phasianus colchicus: 360

Alectoris rufa: 360

Meleagris gallopavo domesticus: 280

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 commercial poultry, the hatching eggs received from breeder flocks are temporarily stored at 18-21 C at the hatchery. Prior to setting in incubators, the eggs go through fumigation, then the temperature gradually increases, and finally, the eggs are set in the incubator at 37 C. After 18 days of rotating incubation, eggs are transferred onto static baskets and set in the hatcher for 3 days. Hatched chicks are removed, selected and vaccinated against certain viruses, then transported to their respective farms. Lorries carrying the chicks must have a controlled environment with adjustable temperature, humidity and ventilation. In this proposed study, for a thorough understanding of infectious diseases

(some are transferred from mother hen to chicks via egg yolk), each of the life stages will be investigated. This includes pre-hatch, hatching and post-hatching monitoring and studying.

Pre-hatch life stage: To date, no work has been done on the impact of single or multiple in ovo vaccination or administration of other substances, such as nutrients or immune-enhancers. This project proposes to sample the embryos at day 18 (the day of in ovo vaccination) and monitor the subsequent impacts on the foetuses at days 19, 20, and 21 (before hatch).

Post-hatch life stage (hatchery to farm): In the poultry industry, soon after hatching and selection of chicks, these birds are given live or inactivated vaccines or nutrients or immune enhancers in the hatchery. In this project, chicks would be sampled before and after this procedure. Such sampling will be continued for certain hours post transportation and upon arrival at the farm. This is to assess the effects of the hatchery processes and transportation on the infectivity and immune induction in the chicks.

Post-hatch (farm/animal experimental facilities): In farm (field) studies, with the farmer's permission, a number of live birds will be randomly sampled for blood or swabs. At specific intervals, a number of birds are sacrificed for a sampling of internal organs, such as the trachea, gut and oviduct, for detection of infection agents, immune responses, microbiome, and proteomic and transcriptomic studies. For control studies, hatched chicks are moved into an animal house in the experimental facilities at the University of Liverpool. Such studies are designed to examine infection, pathogenesis, biology, immunology and vaccine protection.

Abattoirs: These are the final stages of poultry, slaughter or meat of broilers, but for layers-breeders, normally, the meat and other parts will be used for non-human purposes (e.g. pet food). To date, there is no or little study on exacerbation of infection from farm to abattoir due to catching and transportation, and subsequent environmental contamination.

Typically, what will be done to an animal used in your project?

Pre-hatch: Administration of either single or multivalent (live or non-living) vaccines against virus (IBV, aMPV) and mycoplasmas (MG, and MS) via in ovo inoculation of developing embryos at 17-19 (chicken), 21-26 (turkey) and 20-25 (gamebirds) days old. Duration of studies: 3 days in the hatchery and the hatchlings are monitored during transportation, and in the farms. In ovo, injection is delivered into the amniotic cavity (not touching the developing foetus). Thus, there is no pain or distress to the foetus. Billions of hatching eggs are being vaccinated using these techniques globally. The hatchlings normally show no evidence of discomfort.

Post-hatch: Administration of either single or multivalent agents against IBV, aMPV, MG, and MS by spray (aerosol), drinking water, intranasally, oculonasally, in-feed, thoracic air sac, infraorbital sinus, subcutaneously (at loose skin at neck) or intramuscularly (at pectoral muscles). Duration is dependent on the type of bird, e.g. broiler chickens up to 56 days, layers up to 110 weeks, and breeders up to 80 weeks. The procedures are likely to cause temporary discomfort and no lasting harm. Birds may experience some discomfort after the handling and procedure—sometimes resulting in reduced food intake. Full recovery is expected within 1-2 days of the procedure.

Blood sampling. No blood sampling of foetus. Venepuncture is used for chicks, which causes brief pain that does not require analgesics. The volume of blood taken will be small in comparison with total blood volume, TBV (i.e. <5% TBV on any occasion) and should not cause hypovolaemia or anaemia. Normally, depending on the age of the birds, 0.1- 0.5 ml of blood will be collected. A good phlebotomy technique should be adequate to ensure that haematomas occur only rarely. Any birds with excessive bleeding, haematoma or shock will be killed by the Schedule 1 method.

Swabs. The oronasal, oropharyngeal, choanal cleft, intratracheal, cloacal or lachrymal swabs are taken using moistened cotton buds and are associated with brief discomfort for the bird as the swab is inserted and withdrawn. The use of dilute NaCl instilled into the eye may, on occasion, be required to generate a lachrymal response. Birds will be closely observed for any distress, and samples are only taken once.

What are the expected impacts and/or adverse effects for the animals during your project?

The proposed project employs procedures commonly practiced by poultry veterinarians in their routine diagnostic activities. This includes administering vaccines, sampling blood, and taking swabs. Occasionally, a few birds are humanely killed and sampled for tissues.

IBV, aMPV, MG, and MS vaccines are unlikely to induce more than watery eyes in a few birds. After a challenge with virulent viruses or mycoplasmas, clinical signs may appear in unvaccinated birds and perhaps a small number of vaccinated individuals. The virulent strains of IBV, aMPV, MG and MS are, depending on virulence, likely to cause signs of respiratory signs.

Vaccine administration. Small volumes of vaccines (living/non-living) in appropriate carrier solution (about 0.1 ml) are instilled oronasally or oculonasally, which may cause a brief

sneeze or head shake response. For the administration of the vaccines in water or in feed, no adverse reactions are expected. However, birds will be observed for 20 minutes post-procedure. Vector-based vaccines are given in ovo, subcutaneous/wing-web methods. For non-living vaccines, a small volume of non-living vaccine inoculum (about 0.1 to 0.25 ml) will be given subcutaneously/intramuscularly, depending on age group. Though no adverse reactions are expected, all birds will be observed for 20 minutes for any excessive respiratory signs or evidence of shock, including listlessness, depression, tiredness, difficulty in breathing, head tremors and neck/wing/leg paralysis. Any birds with these signs will be killed by the Schedule 1 method. Observation will be continued until the normal behaviour of birds is exhibited.

Inoculation. Small volumes of pathogen(s) solution (about 0.1 ml) are instilled intranasally, oculonasally, intratracheally, intracloacally, subcutaneously, thoracic air sac, infraorbital sinus or intravenously. Based on our publications and literature, the following dosages per bird would be sufficient to achieve the objective of the respective protocols: 104 egg infective dosage (EID)50 for IBV,

103 tracheal organ culture (TOC)50 and 104 colony forming unit (CFU) for MG and MS. For nebulisation, birds will be placed in an aviary chamber and pathogens will be administrated through aerosol. In most instances, there should be no immediate reaction. However, birds will be watched for about 20-30 minutes for any reactions to infection or shock, e.g. sneezing, coughing, excessive headshake, weakness, swelling or bleeding. Any bird with excessive reactions will be immediately killed by a Schedule 1 method.

Swabs. The oronasal, oropharyngeal, choanal cleft, intratracheal, cloacal or lachrymal swabs are taken using moistened cotton buds and are associated with brief discomfort for the bird as the swab is inserted and withdrawn. The use of dilute NaCl instilled into the eye may, on occasion, be required to generate a lachrymal response. Birds will be closely observed for any distress, and samples are only taken once at any point.

Blood sampling. Venepuncture causes some unavoidable pain, which is brief and does not require analgesics. The volume of blood taken will be small in comparison with total blood volume, TBV (i.e. <5% TBV on any occasion) and should not cause hypovolaemia or anaemia. Normally, depending on the age of the birds, 0.1- 0.5 ml blood is collected for birds. A good phlebotomy technique should be adequate to ensure that haematomas rarely occur. Any birds with excessive bleeding, haematoma or shock will be killed by the Schedule 1 method.

Birds under our care are observed no less than twice daily for any signs of discomfort, dullness and depression. Birds with these signs, as well as birds not eating or drinking, will be humanely killed (Schedule 1).

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)?

With the proposed infectious agents of IBV, aMPV, MG and MS, in our experience, our virulent strains cause little to moderate clinical respiratory signs when given in a single infection. This is particularly true in older animals more than 2-3 weeks old. The vaccine strains cause little to no signs in single or co-vaccination. We estimate, about 0-20% of mild clinical signs and 0-5% moderate clinical signs. Our procedures of inoculation, bleeding and swabbing are unlikely to cause any exacerbation of clinical signs.

For the challenge agents of IBV, aMPV, MG and MS, the lowest dosages that causes minimum degree

of disease would be used. In the unvaccinated-challenged birds, mild to moderate severity is expected. No birds would be allowed to progress beyond the moderate severity.

Chicken: mild to moderate (IBV: 80% mild, 20% moderate; aMPV; 100% mild; MG: 90% mild, 10% moderate; MS: 90% mild, 10% moderate)

Turkey: mild to moderate (aMPV; 80% moderate, 20% mild; MG: 80% moderate, 20% mild; MS: 90% moderate, 10% mild)

Gamebirds (pheasants & partridges): mild to moderate (IBV: 90% mild, 10% moderate; aMPV; 90% mild, 10% moderate: MG: 60% mild, 40% moderate; MS: 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?

The proposed studies involve scientific examination of the responses of a living host (poultry) to one or more pathogens/vaccines under an experimental environment. Responses such as host immunity, cellular and tissue injuries, clinical signs and lesions can only be assessed in a living host. At present, there is no other feasible alternative that would replace living birds to achieve the objectives outlined. Host and host-related factors such as natural immune (innate, cellular, mucosal and humoral) responses are part and parcel of the disease mechanism, so using birds is unavoidable. Furthermore, due to the special pathophysiology of birds, it is not advisable to use laboratory rodents or other vertebrates. We have exhausted all other options.

Which non-animal alternatives did you consider for use in this project?

We regularly use the following alternatives to generate some useful data, but fall short of achieving our objectives: -

i. Cell lines such as vero-cells and primary cells prepared from embryos (e.g. chick embryo kidney cells). ii. Fertile embryonated eggs to grow and titration of viruses, e.g. IBV.

Tracheal organ cultures to grow and to titrate viruses, and to provide preliminary virulence (e.g. IBV, AMPV, MG and MS).

Organoids of respiratory/gut epithelium.

Why were they not suitable?

The in vitro and ex vivo non-animal models above provide limited information about immune response details and are unsuitable for vaccination-challenge studies. For an ideal situation, using birds is

advised to study the clinical signs, pathology, and vaccination-challenge efficacies. To align our outputs with the requirements of international organisations such as European Pharmacopoeia, the use of birds became unavoidable for vaccination-challenge 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 number of birds was decided on consideration of the 3Rs, requirements of European

Pharmacopeia and statistically sound approaches. In each experiment will consist of many groups. From each group, 6-10 birds will be randomly sampled at each sampling point. Based on our past publications and literature reviews, with the lowest titre of IBV, aMPV, MG or MS inocula dosages, the studies' objectives can be achieved by including at least 6 birds per replicate. Based on variance analysis, a group size of 6 birds/group for parameters of antibody or antigen titre differences between the groups can be analysed at 80% power and 5% significance. Thus, 6 birds per group will be used, except for when up to 10 is required by the European Pharmacopeia.

All experiments are thoroughly realistic, high-quality, and cater to the objectives of this project application. I am determined to maintain high care and optimal management and fulfil all welfare requirements of the birds while attempting to generate essential, highly scientific data.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

I have been working on in vivo experiments, such as designing, execution, data analysis and optimization of outputs, since 1994. I will closely liaise with our statisticians and epidemiologists at out institution on experimental design and statistical analysis, especially to keep the number of birds to a minimum. The proposed experimental protocols will likely generate nonparametric and parametric data for the mortality, clinical signs, lesions scores and number of birds with antigen/antibody detection.

The experimental design will consider sampling 6-10 birds from each group at each sampling point. The birds will be randomly allocated. Birds will be sampled randomly (using wing band number) on sampling days. This will allow required statistics to be gathered and requirements of vaccine registration organisations, such as the European Pharmacopeia, to be fulfilled.

The data would be analysed using Fisher’s exact or Chi-square test, Kruskal-Wallis or Mann-Whitney U test. Parametric data, such as body weight, serological titres, and others, would be analysed using a one-way analysis of variance to compare between the groups, followed by Tukey’s post hoc, Dunnet’s or student-t test. Statistical significance

would be assessed at $p<0.05$ for all comparisons. University statistical software SPSS, Minitab, or R 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?

In each experiment, where possible, we ensure that multiple objectives can be fulfilled in a single animal experiment. For example, clinical assessment is accompanied by pathological evaluation of different tissues. We collect many different tissues and body fluids.

Beyond the experimental design, where possible, we assess the pathogens/vaccines in vitro, for example, using tracheal organ cultures (from 18-day-old embryos). Each embryo can provide 10-15 tracheal rings. We have assessed the virulence of vaccine viruses using tracheal rings. This provides preliminary details for reducing the number of birds in experimental 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.

Chickens, turkeys and gamebirds will be used because they are the most susceptible hosts to the diseases under study and are important and common farmed birds in the UK. Chickens and turkeys are the most susceptible to IBV and aMPV. For MS, chickens are the most susceptible host. MG can cause similar levels of disease in chickens, turkeys, and gamebirds.

The respiratory pathogens cause relatively mild infections, which normally resolve in 7-10 days. The commercial vaccines cause no distress. All birds will be monitored daily. Numbers in experimental groups will be kept to a minimum, consistent with producing meaningful results and allowing for individual variation.

The birds are always kept in the best environment, where floor space, ventilation, light and lighting, feed and feeding, water and watering, behavioural needs (e.g., perching), and other requirements are provided to optimal standards. Any birds with welfare concerns, either due to health or otherwise, would be put to sleep humanely.

Why can’t you use animals that are less sentient?

When essential, we will move on to the use of live poultry. IBV, aMPV, MG, and MS are diseases specific to domestic poultry and to assess a virulent strain of pathogens or vaccines, it is highly recommended to use living whole birds. This is as host-antigen interactions, particularly pathogenesis and immune responses, could be thoroughly studied. As such, it is essential to use host poultry when absolutely needed. For vaccine assessment, protection studies in the respective host provide undisputable results and are

accepted by authorities (e.g. European pharmacopoeia), scientists, veterinarians and producers worldwide.

Poultry vaccines of IBV, aMPV, MG and MS are administered at 18-days of incubation, day-old or older birds. As such, our work must mimic the industry practice. Comparable publications are available for infection, immunity, diagnosis, epidemiology and vaccinology of these organisms in poultry.

IBV, aMPV, MG, and MS only infect avian hosts. IBV, especially, is only known to cause disease in chickens. aMPV in chickens, turkeys, and gamebirds. MG in chickens, turkeys, and gamebirds. MS in chickens and turkeys only. As such, the avian host is essential for the proposed studies.

To date, infectivity and/or disease due to IBV, aMPV, MG or MS has not been demonstrated in rodents or other experimental animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Chicken is the only natural host of IBV, and for aMPV, MG, and MS, turkeys, chickens, and gamebirds are susceptible. For experimental infection, as outlined by global vaccine registration authorities – USDA and European Pharmacopeia, chickens and turkeys will be used. As an expert scientist in IBV, AMPV, MG, and MS, the objective of the studies can be achieved by limiting the severity to mild and moderate.

With my experience as a poultry veterinarian and researcher, animal suffering will be minimised in two ways: -

Prevention of excessive disease manifestation. This is done by using the lowest dosage of inoculum where only a milder form of disease is produced, which is sufficient to achieve the objective of the work.

Defined endpoints that are clear and simple for everyone to follow. Based on my experience as a poultry veterinarian and researcher, clinical signs normally produced with pathogens listed above in the respective species are outlined and made available in each experimental room. When clinical signs or scores are recognised, the NACWO or I will be approached for immediate assessment. To facilitate a quick decision, clinical scores and actions are listed below. Birds with a score of 5, will be kept under increased observation, and those with a score of 6 will be humanely killed.

General and respiratory signs (all pathogens) Clinical score For IBV, MG and MS normal 0

sneezing/snicking 1 watery eyes 2 nasal/ocular discharges 3 audible tracheal rales 4 huddling/ruffled feathers/depression plus 1-4 5 laboured breathing 6

For aMPV inoculated birds only

normal 0

clear nasal exudate only or frothy eyes only 1 turbid nasal exudate 2 swollen infraorbital sinus or frothy eyes and nasal exudate 3

3. The room temperature, air quality, hygiene and sanitation, floor/feed/water space requirements will be maintained to the required standards. Birds that are unable to move, drink or eat, or are distressed, will be humanely killed by Schedule 1 methods.

Stringent inspection schedules, including increased visits, are always implemented to ensure the welfare of birds is closely monitored and protected.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Chickens and turkeys will be used because they are the most susceptible hosts to IBV and aMPV, respectively. Chickens are most susceptible to MS. MG causes a similar degree of disease in chickens, turkeys, and gamebirds. Most importantly, these are the most common and important farmed birds in the UK.

The respiratory pathogens to be used cause relatively mild infections, which normally resolve in about 7-10 days. The commercial vaccines cause no distress. All birds will be monitored daily. Numbers in experimental groups will be kept to a minimum, consistent with producing meaningful results and allowing for individual variation.

At all times, the birds are kept in the best environment, where floor space, ventilation, light and lighting, feed and feeding, water and watering, behavioural needs (e.g., perching), and other requirements are provided to optimal standards. Any birds with welfare concerns, either due to health or not, are put to sleep humanely.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

As a project and personal licence holder, I keep myself updated with 3Rs. For new developments in 3Rs, I receive emails from our Home Office veterinarian, receive regular updates via the NC3Rs enewsletter, attend ad-hoc NC3R University or third-party online webinars.

87. Using behaviour to understand fish welfare

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

Behaviour, Environmental change, Fish, Stress, Welfare

Animal types	Life stages
Zebra fish (Danio rerio)	Embryo and egg, Neonate, Juvenile, Adult
Teleost fishes	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult
Brown Trout (Salmo Trutta)	Embryo and egg, Neonate, Juvenile, Adult
Salmon (Salmo salar)	Embryo and egg, Neonate, Juvenile, Adult
Rainbow Trout (Oncorhynchus mykiss)	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?

To understand how fish behaviour and welfare is influenced by environmental factors in both artificial (e.g. ornamental fish trade, aquaculture) and natural contexts.

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?

Under commercial settings, fishes can be exposed to a range of stressors; in order to protect fish welfare, and indeed promote positive welfare, we need to understand how environmental change (both human-induced and natural), affects fish behaviour and physiology (Objective 1 of this project). For example, if fish are transported live within the ornamental trade or in aquaculture, changes in water quality during transport can lead to increased stress and compromise fish welfare. Similarly, low levels of contaminants within the natural environment can cause subtle changes in fish behaviour that can have serious implications at the population level if they interfere with the ability of fishes to catch prey or avoid predation. Understanding how fish behaviour and physiology are influenced by environmental factors can therefore help improve the welfare of fishes, including during sensitive early life stages and across generations. Traditionally, monitoring the way fishes respond to environmental change has utilised invasive or terminal sampling; therefore, alongside this need for fundamental knowledge, we aim to develop robust non-invasive, or minimally-invasive, measures of stress in fishes (Objective 2 of this project).

What outputs do you think you will see at the end of this project?

Outputs from this project will include new information on the key environmental factors which affect behaviour and welfare of fishes, including ways to mitigate these effects. This information is crucial for determining the best ways to protect their welfare and protecting natural populations. New information will be made available primarily through peer-reviewed publications, industry reports, scientific conferences and public presentations.

Who or what will benefit from these outputs, and how?

Within the time-frame of the proposed work, the welfare of fishes within commercial practices will benefit from research findings that identify specific refinements to current practice. For example, changes in the way fishes are transported may be identified that can be shared both directly with industry through reports, or more widely through peer-reviewed publications and conference presentations.

In the medium term, developing a suite of refinements for fish welfare in commercial practice, coupled with developing methods to non-invasively assess fish welfare (e.g. observing behaviour, measuring hormones released into the water and taking mucus samples) will strengthen our ability to improve fish welfare across a range of scenarios.

A greater understanding of how environmental factors influence fish behaviour and welfare will allow development of this field of research to ensure long-term improvements in the welfare of fishes held in ornamental trade and food-fish aquaculture. This new information will help develop mitigation strategies and also has importance in conserving natural fish populations.

How will you look to maximise the outputs of this work?

Our research group works closely with industry (for example with wholesalers and retailers of ornamental fishes and food fish aquaculture companies) and therefore can ensure that results are disseminated in a timely fashion, often considerably prior to publication in the peer-reviewed literature. Unsuccessful approaches are also discussed with our industrial partners so that refinement of approaches, and consideration of ‘non-significant’ effects occurs.

Species and numbers of animals expected to be used

Other fish:

Teleost fishes: 4500

Zebra fish (Danio rerio): 1000

Brown Trout (Salmo Trutta): 1000

Rainbow Trout (Oncorhynchus mykiss): 1000

Salmon (Salmo salar): 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.

The fish species used in this project will be mainly freshwater fish species that are either considered to be model species for fish behaviour and physiology (e.g. zebrafish) or are important commercial fish species (both for food-fish aquaculture and the ornamental fish trade). It is important to understand the response of all life stages of fishes to husbandry practices and environmental change; indeed we know that husbandry conditions of female fishes can affect subsequent behaviour of their offspring. Therefore, all life stages will be considered.

Typically, what will be done to an animal used in your project?

Typically, fishes will first be exposed to a change in environment which may be procedural as it has the potential to cause an adverse effect (e.g. provision of a diet with a lower than normal level of protein, experience of simulated transport) or non-procedural (e.g. addition of environmental enrichment).

Following this change in environment, fishes will be monitored for changes in behaviour and physiology. Behavioural measurements may include: ability to perform in a maze environment

(procedural), to compete/socially interact with other individuals (procedural), and other non-procedural measures including activity, feeding, interaction with enrichment. Physiological measurements could include: mucus sampling (procedural), blood sampling (procedural), respirometry and/or swimming ability (procedural) and other non-procedural measures including water-borne hormone levels, body colouration.

At the end of an experiment, fishes may be released from the act and rehomed if they are not suffering, or likely to suffer, any adverse effects of regulated procedures. This will be determined by a veterinary surgeon or a competent person using criteria agreed with the named veterinary surgeon in line with standard project licence conditions.

What are the expected impacts and/or adverse effects for the animals during your project?

Potential (rather than expected) adverse effects from this project could include adverse changes in behaviour (e.g. lack of feeding, loss of equilibrium/swimming ability, laboured breathing i.e. increased or irregular movements of the opercula) and skin abrasions. Any fish showing signs of these adverse changes will be immediately removed from the experiment and euthanised by a Schedule 1 method if recovery is unlikely, thus the duration of these effects would be minimal.

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)?

With the exception of fishes exposed to aquatic toxicants (5%) the expected severity of procedures is mild. The work involves exposing fishes to mild stresses that they would naturally encounter in the wild or in a commercial environment (e.g. in aquaculture or as companion animals). For those fishes exposed to aquatic toxicants, very low levels of contaminants are used to look for subtle changes in behaviour and physiology, therefore it is unlikely that adverse effects will be seen. However, due to the nature of these experiments they are classified as moderate. It is anticipated that the majority (95%) of fishes from this project that have only been exposed to mild stresses will be released from the act either to stock or re-homed as companion animals where possible.

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?

Currently there are no *in vitro* or computer-based alternatives for this type of research. We have used the www.frame.org.uk website and associated links to look for alternatives and can find none documented but will continue to monitor both this website and the scientific literature to look for alternatives. Where possible we use non-invasive measures (e.g. behavioural observations of fishes during commercial practice) to address our objectives.

Which non-animal alternatives did you consider for use in this project?

There are no *in vitro* or computer-based alternatives for this type of research.

Why were they not suitable?

There are no *in vitro* or computer-based alternatives for this type of 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?

For all experiments associated with this project, experimental design is based on statistical planning. Careful consideration of existing literature, including our own unpublished and published data is used to inform experimental design. Tank replication has also been considered as there may be variability caused by different social groups within tanks. This allows us to use the minimum number of fishes while ensuring statistical robustness.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Consultation with statisticians (in-house and/or industry based) occurs both during experimental design and of the final experimental design prior to the start of experiments. For research involving industrial partners, additional statistician input to data analysis approaches allows us to continuously refine our experimental designs and to minimise the number of fishes being used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For work with embryos, many measurements can be made prior to hatch with only ~25% of embryos used raised to first feeding and becoming licensed fishes. Therefore, some partial replacement occurs. Additionally, we continue to develop non-invasive methods of measuring fish welfare, and to work with industry to monitor and refine fish welfare under existing commercial practice, thus reducing the need for experimental research on fishes.

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 this project will be typical teleost fishes (mainly freshwater) that are either considered to be model species for fish behaviour and physiology (e.g. zebrafish and salmonids) or are important commercial species (both for food-fish aquaculture and

the ornamental trade). These will include species from the families Goodeidae and Cichlidae among others.

For each objective and experiment, appropriate fish species will be chosen based on sound scientific reasoning. Many of the research questions posed in this proposal are related to ornamental fish species and in considering social behaviours and responses to husbandry stressors it will be necessary to consider species with a range of life histories, such as live-bearers and egg-laying species.

In the protocols, different methods of carrying out a procedure are given where different species may require different procedures. Where a choice of methods exists, the mildest method to achieve the stated objectives will be chosen.

Why can’t you use animals that are less sentient?

Our previous work has refined the use of techniques to assess the impact of environmental change on very early life stages, thus reducing the need to raise embryos beyond first feeding. We will continue to use these methods where they can appropriately address the objectives. However, to understand impacts of husbandry and environmental stressors on fish behaviour, it is not possible to use species that have been terminally anaesthetised or are less sentient.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Welfare costs associated with this project are expected to be mild. Fish health and welfare will be monitored on a daily basis by experienced staff (e.g. the NACWO) including feeding behaviour, swimming behaviour and overall condition of fishes. Water quality parameters are checked on a daily basis.

Protocols 2 and 3 (of 3) are mild and chosen for their sensitivity in detecting differences in physiology and behaviour. Protocol 1 is classed as moderate due to the potential exposure to aquatic toxicants (N.B. this is an optional component of this protocol). Only mild toxicant effects are anticipated in line with the overall objective of understanding the effects of sub-lethal doses. There is an extensive body of literature documenting the effects of aquatic contaminants on fishes, particularly with regard to lethal and acutely toxic concentrations. Here the effects of chronic exposure (ideally at the lowest observable effect concentrations) are of interest and suitable concentrations will be calculated by carefully scaling down concentrations known to be acutely toxic. For all protocols, where a choice of methods exists, the mildest method to achieve the stated objectives will be chosen.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We use a variety of practice guidance to ensure refinement of experimental techniques. These include, but are not limited to:

Guidelines for the treatment of animals in behavioural research and teaching (ASAB, 2012, 2018).

Ethical justification for the use and treatment of fishes in research (Journal of Fish Biology, 2006; Metcalfe & Craig, 2011).

Guidelines for the use of fishes in research (AFS, 2014).

The ARRIVE guidelines 2.0: updated guidelines for reporting animal research (Percie du Sert et al. 2020).

PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) Guidelines (Smith et al., 2017).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We regularly consider updates on the NC3Rs website and receive information through AWERB meetings and email communications. I attend meetings with the ethics teams of industrial partners. Where advances in the 3Rs, particularly with regards to refinement occur, we look to implement these within our work. A recent example would be redevelopment of our lighting system to ensure a smoother on/off transition. Both PI and students have previously and will continue to attend LASA, RSPCA and NC3R events, including those specifically focused on fish research. These include recent events such as RSPCA Focus on Fish (2025). I am invited to attend the Fish Vet Society event (March 2025) which includes discussion on fish welfare, including ornamentals. I am also an external member of the University of Gibraltar Research Ethics Committee which facilitates discussion on the 3Rs at an international level.

88. Form and function during mammalian heart development

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

Heart development, Embryogenesis, Cardiac Physiology, Regeneration

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 seeks to understand how the mammalian heart forms during embryonic development, focusing on mechanisms by which form and function emerge and interact. If we can understand how the heart forms in the first place, then we will be better equipped to repair or replace it when it fails during 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?

It is important to undertake this work in order to address questions of fundamental biological importance but that also have clear clinical relevance. This relevance includes:

Congenital Heart Defects (CHD). CHDs are the most common type of birth defect, affecting 1:150 babies born in the UK (13 a day), however for over half of these cases there is an unknown cause. By studying how the heart forms we will gain new

understanding into the mechanisms which regulate its development and thus identify new causes of CHD.

Regeneration. During a heart attack, billions of cells are lost from the heart leading to death or heart failure. Heart attacks cause 1.8 million deaths each year in Europe alone and whilst more people are surviving a heart attack those surviving subsequently go on to live with heart failure, another debilitating disease. In order to prevent this heart failure therapies are being developed which look to replace the lost cell by creating and implanting heart cells made from stem cells. In order to make the right cell type it is important we understand how to accurately make these cell types and therefore developmental biology acts as instruction manual in regards to how to make these cell types. Another regenerative approach is to reactivate mechanisms which cause the heart to grow in the embryo in the adult in order to stimulate new growth. If we can understand how the heart forms in the first place, we are better equipped to repair or replace it when it fails.

Cell based models of disease. To reduce animal experimentation and also gain biologically relevant human insight, it is important that we have reproducible cell based models. These models are based on using stem cells and can reflect at the cellular level what happens during embryo development. To generate accurate cell based models we need to understand how development happens in the embryo proper. Whilst our understanding of human heart development is increasing, human samples are extremely rare, thus it is fundamental we use model organisms to understand how the heart develops at an organ level. Once we have organ level reference data we can then refine and better trust the cell based models before using them to gain human relevant insight, such as understanding mechanisms of disease. For example, during heart disease there is an increase in the expression of genes which are required for heart development, therefore by studying developmental processes and gene regulation in the embryo we can identify ways in which we can control these genes during disease.

What outputs do you think you will see at the end of this project?

At the end of this project we will have generated publications describing new insight into mechanisms controlling heart development and pathways which could play a role in diseases such as CHDs, heart failure, irregular heartbeat. This work will also highlight strategies in which we can create new cells to repair the loss of cells which occurs during a heart attack, thus aiding regenerative approaches.

Who or what will benefit from these outputs, and how?

Short-term outputs will benefit the scientific community by providing novel understanding in regards to form and function during heart development. Longer-term outputs will aid with the development of strategies to treat disease. Through the dissemination of our work at public events, such as Science festivals, we hope to increase understanding of our work with the general public.

How will you look to maximise the outputs of this work?

To maximise the output of our work we will disseminate our work at conferences, science festivals and publications in open access journals. Datasets will be made publicly available at time of publication in accordance with the principles of our academic institution. Through collaboration we will also maximise the potential from our work by sharing new tools and samples. We will also publish/make publicly available any unsuccessful approaches to aid the community.

Species and numbers of animals expected to be used

- Mice: 5250

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 appropriate mammalian species to be used in this project due to the similarities they share with humans in regards to heart development (i.e. cardiac morphology 4-chambered heart). Moreover, mice are a well characterised and widely used model of mammalian embryo development. Another advantage of using mice as a mammalian model is the availability of transgenic animals not available in other model mammalian species such as rats.

Adult mice will be required to produce, maintain and generate genetically altered embryos. Our research aims to explore how the embryonic heart develops, thus we will require pregnant females to collect or label embryos. Using both embryos and adult mice we will examine heart shape in different strains of mice and explore how cells in early development contribute to the embryonic and adult heart. To generate new genetically altered mice we will require the collection and use of mouse eggs for fertilisation, microinjection and implantation. Neonate and Juvenile mice will be used to examine heart shape and how distinct embryonic cells contribute to the heart post-birth and prior to adulthood.

Typically, what will be done to an animal used in your project?

The main procedure performed in this project will be the breeding and maintenance of transgenic animals and the generation of embryos from timed matings. Embryos will typically be collected from pregnant females, during early development between embryonic day (E) 6.5 and 16.5. This will involve the pairing of female mice with a singly-housed stud males late in the afternoon. The following morning vaginal plugs will be check and any plugged females will be separated, and typically placed with another female to prevent singly housed animals.

We will perform intraperitoneal (into the abdominal cavity (IP)) injections to label/modulate cells during development. Typically this will require a single IP injection to a pregnant female during early development with embryos being collected before birth. As we will humanely kill the female to recover embryos this will typically only happen once to a pregnant female. Administration of labelling agents will also be administered either via oral (addition to drinking water/food), IP or gavage (feeding tube direct to the stomach) administration.

We will also perform temporary anaesthesia on pregnant females in order to conduct embryonic ultrasound recordings. Pregnant females will receive inhalant anaesthetic and be maintained on a heated stage with monitoring of heart rate. Whilst under anaesthesia ultrasound will be performed to characterise embryonic heart shape and heart rate which should require less than 45mins of anaesthesia. Having completed recordings the females will then either be humanly killed or allowed to recover. Following recovery embryos will either be collected at subsequent stages of development or allowed to litter down. This procedure would typically happen once to a pregnant female. A maximum of two recordings may be taken but the second recording would be non-recovery anaesthesia.

During non-recovery ultrasound we may administer substances to modulate heart function whilst under anaesthesia e.g. isoproterenol. These substances would be given either into a vein or intraperitoneally.

What are the expected impacts and/or adverse effects for the animals during your project?

The adverse effects experienced by the mice will typically be minimal. Intraperitoneal injections will cause mild transient pain during administration and oral garage will increase stress but should not be painful. In some cases the substances administered may lead to temporary weight loss, this will be for a limited duration as we will typically be working with embryos and the pregnant female will be humanely killed 4-8 days following 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)?

Mice:

- Subthreshold = 74%
- Mild = 25%
- Moderate = 1%
- Severe = 0%

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?

As our research focuses on the development and function of the mammalian heart during embryonic development. In order to understand how form and function arises during mammalian development, it is essential that live, intact embryos are used and therefore animal use is necessary. The proposed research aims to understand how the heart starts to beat and what influence this beating has on the development of heart shape and cell type composition. Due to the complex shape changes which the heart undergoes during development, the multiple different cell types which are required for its function and the signalling that occur in the intact embryo, such experiments can't currently be conducted in an cell-based or computer models.

Which non-animal alternatives did you consider for use in this project?

We have considered and are using cell based models, human embryos and computer modelling as alternatives. These will reduce experiments conducted in mice. However these cannot yet replace the complexity of embryo development and human samples are extremely rare at these stages and have increased ethical implications.

Why were they not suitable?

To gain human relevant insight, human embryos would be the most suitable model to study. This, of course, is ethically challenging, and extremely limited due to early stage of development at which the human heart forms. We must therefore rely on standard animal model systems like the mouse. Cell based approaches will be used to look at how human stem cells can form different types of heart cells in 2D and 3D. This will allow use to explore specific cellular mechanisms and increase the relevance of our research as well reduce the number of animals used; however, these models do not accurately copy/model the multicellular 3D processes that occur in the intact embryo. Thus, we require the use of mice to make direct observations and manipulations in intact embryos in order to provide a benchmark for the cell-based models we plan to develop.

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 has been calculated based on the proposed number colonies we will require, our experimental plans (mainly relating to the generation of embryos from timed mating) and my 13-years of mouse colony management experience. Of the 5250 mice we estimate that 1000 mice will be used for experimental protocols (everything other than breeding and maintaince). However of the 4250 mice not used for experimental protocols around 40% of these will be used to generate embryos for experiments.

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 in this project we will take a number of steps.

Initially before moving to mice we will use previously published datasets to determine feasibility or cellbased models using human stem cells to refine our hypotheses, thus minimising the experimental questions requiring animal studies as well as maximising the focus and information obtained. We are also part of the NC3Rs cardiovascular network, which will provide opportunities to explore new biologically-relevant cell based models. We will use the PREPARE guidelines prior to initiating any experimental study to aid in the planning of each stage, and the ARRIVE guidelines to help in the design, analysis and reporting of all studies.

Longitudinal in utero ultrasound monitoring of embryonic heart development will provide data relating to both function (heart rate, blood flow) and form (heart size and morphology) in a non-invasive manner. This approach will allow multiple parameters to be measured from an entire litter

(approximately 8 embryos) using a single animal without the need to kill the animals at each time point thus reducing the numbers of animals needed per experiment.

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 strategies - to reduce the number of animals used for breeding we will manage our colonies actively with regular monitoring and varying colony sizes depending on current experimental plans. To optimise the number of animals used we will manage our colonies interchangeably e.g. rather than ordering in fresh normal wild type mice (for breeding or experiments, we will use wild type mice generated from another one of our colonies. Where possible we will use breeding strategies which generate litters in which all animals have two copies of the required allele (homozygous), this will mean that all mice generated can be used for experiments or breeding. In other situations this breeding will not be possible due to early embryonic lethality when mice are homozygous, and thus these colonies will have to be maintained with a single allele (heterozygous) to enable breeding. We will use an internal tissue sharing distribution list to share mice due to unwanted genotypes or identify animals which could be used for experiments that would otherwise be culled. Randomisation will occur due to the varying genotypes of embryos within a single litter, meaning experiments will have inherent randomisation.

Sharing of tissue - given we focus on heart development, there are a number of other research groups in our institute who work on different organ systems during development such as the brain, blood and limbs. We will therefore share other parts of the embryos with these groups to optimise usage and reduce the number of mice generated.

Pilot studies - when establishing a new experimental plan we will use pilot studies to first determine whether we can detect any measurable differences. This will allow us to determine whether it is experimentally valid to continue with the proposed plan e.g. calculate sample sizes, determine severity level. Studies will be completed using randomisation and blinding.

We will also take advantage of our colony management system in order to identify where possible colonies that are already present within our facility and therefore reduce us importing/generating new animals. I am also part of the establishments colony management group that share best practice and guidance.

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.

Most of our methodology will be timed mating to generate embryos and thus animals will experience sub-threshold levels of pain and suffering. In some instances the animals will experience mild transient pain due to the administration of substances however this will be temporary. For genotyping we will use the most refined method possible, typically this will be ear biopsy which will cause the least amount of pain and also generate enough

material for accurate result generation. Intraperitoneal injections of tamoxifen will be performed on adult and pregnant mice, which will cause mild transient pain but is required in order to provide rapid administration and generate results with biological relevance. If administration of tamoxifen is required in neonates we will look at using a more refined method such as orally from the end of a syringe. We will always liaise closely with our animal facility team and user management group to identify the current most refined approach.

Why can’t you use animals that are less sentient?

Whilst other model species such as fish, toads, and flies do have hearts they have a different anatomy, which does not reflect the four chambered mammalian heart. They also undergo different process during embryonic development and have different mechanisms by which they regulate the heart beat. As we are exploring mechanisms which relate to human congenital heart defects it is fundamental that we work with a model system which is as similar as possible to the human. Mice are the most appropriate mammalian species to be used in this project due to the similarities they share with humans in regards to heart development (i.e. cardiac morphology 4-chambered heart). Moreover, mice are a well characterised and widely used model of mammalian embryo development. Another advantage of using mice as a mammalian model is the availability of transgenic animals not available in other model mammalian species such as rats. Mice are the least sentient models that we can utilise to address our experimental objectives in a biologically relevant manner and develop therapeutic relevance.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The majority of the procedures that we will perform have been extensively refined. For pregnant females we will aim to reduce all stress which will require a balance between monitoring the animals and leaving uninterrupted following timed mating. To reduced stress further we will refine our protocols for example with using tunnel handling. For the treatment of animals with tamoxifen as well as other substances we will monitor the best administration approach (e.g. IP injection, gavage), following internal tamoxifen administration guidance and liaising with Named Persons. Areas of refinement could include implementing a welfare scoring sheet following tamoxifen administration. We will also use a heated stage and warm gel when using ultrasound scanning. If animals do show signs of weight loss we will provide heat packs and soft food or a high-calorie diet to provide additional support.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We are regularly informed of the NC3Rs latest initiatives and publications by our establishment's HO licensing team. Our animal facility also has regular colony management and user meetings, where we are informed about current best practice. We will follow the PREPARE guidelines (Smith et al. 2018) as well as use the reporting metrics detailed in ARRIVE 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?

I am part of the NC3R cardiovascular network, and recently attended the launch event in London. We will attend Regional 3Rs symposia and other local webinars provided through our establishment. Our establishment also hosts colony management meetings and training events which provide information regarding the 3Rs and best practice. Another

valuable resource will be our Named Persons, including the Establishment Licence Holder, Named Information Officer (NIO), Named Animal Care and Welfare Officer (NACWO) as well as Named Training and Competency Officers.

89. Influenza virus research in mammalian species

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

influenza, virus, swine, ruminants, livestock

Animal types	Life stages
Embryonic forms: Avian embryonated chicken eggs	Embryo and egg
Cattle	Juvenile, Adult
Pigs	Juvenile, Adult
Alpaca	Juvenile, Adult
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 this licence is to safeguard animal health and welfare by researching influenza virus infection in mammals, particularly livestock species, in order to limit the impact of disease. This licence will facilitate study of viral infection dynamics, virus transmission mechanisms and the host response to infection, including development of immunity. Additionally, this licence will support research activities for viruses of concern, such as novel emergent strains, to assess risk pathways and inform intervention strategies, such

as vaccine deployment. Infection of embryonated chicken eggs with mammalian-origin viruses will also be required for virus isolation, propagation and assessment of infectivity.

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?

Animal influenza virus infection has a detrimental impact on animal health, agriculture, food security and the economy. Additionally, influenza viruses are able to spill over and cause infection in different host species, including humans, with associated One Health implications. Pandemic influenza is included in the UK National Risk Register (NRR) of Civil emergencies. This zoonotic (animal to human transmission) risk was clearly demonstrated by the 2009 H1N1 pandemic in humans and panzootic in pigs. A human infection caused by a swine-origin H1N2 virus was also detected in the UK in Nov 2023. More recently, highly pathogenic avian influenza H5N1 viruses have been shown to infect diverse mammalian host species including marine mammals and wild carnivores. In the USA, this avian-origin virus is causing epizootic infection of lactating dairy cattle and human occupational infection. This heightened influenza threat highlights the importance of research capability to investigate mammalian influenza virus infection of the biologically relevant livestock host species. In particular, swine influenza is closely monitored at a global level and the UK contributes biannually to the report on swine influenza A virus status submitted to the World Health Organisation (WHO) by the swine influenza subgroup of the OFFLU (World Organisation for Animal Health (WOAH) and Food and Agriculture Organization (FAO)) joint influenza committee.

This licence sustains the essential capability to safeguard the UK by supporting research into animal influenza viruses that are constantly evolving and potentially pose significant risks, such as the ability to cause epizootic disease outbreaks in livestock species and pandemics in human populations. It will also allow scientific knowledge gaps to be addressed for newly described or (re-)emerging influenza viruses, including influenza D virus (IDV) as well as infection dynamics in less well studied host species, such as cattle and camelids. Additionally, this licence will support reactive research in response to emerging influenza threats, such as the identification of new viral variants with unknown virological and pathogenesis profiles. The ability to conduct influenza research in an agricultural setting (POLE) is especially important in this context to allow monitoring of unusual outbreak events in farmed herds.

What outputs do you think you will see at the end of this project?

The key objective of this licence is to support research into influenza virus infection in mammalian livestock species to increase scientific knowledge concerning disease dynamics as well as to inform risk prediction and pandemic preparedness activities. The main outputs will include scientific reports, publications and presentations relating to new research findings, including characterisation of novel and (re-)emerging influenza disease threats as well as assessment of possible control and mitigation strategies. Furthermore, research data will also be used to provide scientific evidence to inform assessments of mammalian influenza risk to both Animal and Public Health Agencies and provide policy-relevant information to UK and international stakeholders.

Who or what will benefit from these outputs, and how?

The fundamental purpose of this licence is to support an internationally recognised mammalian influenza research programme and to disseminate research findings. Research outputs are essential in order to promote continued engagement with numerous potential beneficiaries including the scientific community, veterinarians, farmers, the pork and Biopharma industries as well as other competent authorities and stakeholders. Research may also be conducted as a rapid response in reactions to influenza disease events. These activities will not only benefit animal health and welfare but will also provide the capability to inform potential influenza pandemic risk and response strategies

Furthermore, through the UK national and international influenza reference laboratory networks and organisations such as the European swine influenza (ESFLU) network of excellence, these research outputs will also provide policy-relevant information to inform both national and international stakeholders. These stakeholders include competent authorities such as the UK Department for the Environment, Food and Rural Affairs (Defra), the World Organisation for Animal Health (WOAH) and the WOAH-Food and Agriculture Organisation (FAO) network, OFFLU, that reports biannually to the WHO regarding animal influenza risks.

How will you look to maximise the outputs of this work?

The research programme will communicate new scientific knowledge through scientific reports, publications and presentations. Furthermore, this licence supports the research outputs that are disseminated through the UK National and International reference laboratory networks and organisations. This includes the sharing of virus and reagent panels, including hyperimmune antiserum. Significant research findings are communicated to competent authorities and policy stakeholders such as Defra through the UK Pig Expert Group and Veterinary Risk Group and equivalent government departments world-wide. Relevant findings are also disseminated through membership of International networks including Academia, the European swine influenza network, ESFLU, the World organisation for Animal Health (WOAH), the WOAH-Food and Agriculture Organisation (FAO) influenza subgroups for influenza (OFFLU) and the European swine influenza (ESFLU) network of excellence.

Species and numbers of animals expected to be used

Other birds:

Embryonic forms: Avian embryonated chicken eggs: 500

Cattle: 290 Pigs: 650 Camelids:

Alpaca: 130

Sheep: 290

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 choice of species reflects the biologically relevant host appropriate for the viruses being studied and the scientific questions to be addressed. Where possible, an alternative to live animal studies is sought, for example laboratory studies using cell or organ culture

or less sentient embryonic forms (avian embryonated eggs). However, it is not always possible to replace live animals when addressing certain scientific questions, for example when investigating complex virus-host interactions that occur during viral transmission or the host immune response to infection.

Typically, what will be done to an animal used in your project?

The majority of procedures to be done on animals are sampling and administration of substances, with or without anaesthetic. The majority of sampling procedures will require daily swabbing of external orifices over a maximum continuous 28 day period eg swabbing of the nasal cavity. Blood sampling from a superficial vein (such as the jugular vein) will normally be done at weekly or longer intervals but in rare cases, with a minimum daily interval. Volumes obtained will be within prescribed limits in accordance with NC3Rs principles Blood sampling: General principles | NC3Rs. Substances most likely to be administered include virus suspensions inoculated into the nasal cavity (maximum of two occasions) or immunogens (e.g. vaccines or antigens) delivered either by injection or instillation (maximum of 4 occasions). Some studies (e.g. virus transmission or infectivity in the environment) will require introduction of animals into a virus-contaminated environment or co-housing infected animals with healthy contact animals to assess transmission. Optional peri-mortem procedures may include exsanguination under deep terminal anaesthesia. Euthanasia will be done using a Schedule 1 method. This licence will also use embryonated chicken eggs, that are less sentient than hatched animals, for influenza virus inoculation to allow virus isolation and propagation.

What are the expected impacts and/or adverse effects for the animals during your project?

Adverse effects of research procedures as a result of restraint during sampling or inoculation are anticipated to be mild and transient on the majority of occasions. Anaesthesia will be applied during procedures if the administration of anaesthetic is not more severe than the procedure itself.

The main potential adverse effects are expected to be clinical signs resulting from infection following virus inoculation. Research studies frequently have the objective of characterising novel virus strains, or virus infection in novel host species, so the expected adverse effects are not known. Clinical signs of influenza are mainly observed in a field setting and normally manifest as acute respiratory disease that normally resolves within a week. Signs may include nasal discharge and fever but may also be associated with coughing, laboured breathing, lethargy and weight loss. Experimental influenza infection studies conducted under previous licences have resulted in inapparent or mild, transient clinical signs and very few animals have reached moderate severity. Species-specific clinical score sheets have been refined during these previous studies and are applied to ensure standardised clinical monitoring, limit the duration of clinical signs and define humane endpoint criteria. Intervention criteria are specified, including the use of treatment as advised by a NVS eg analgesic medication to reduce fever.

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)?

Embryonated chicken eggs

Sub-threshold 100%

Pigs

Mild 98%

Moderate 2%

Cattle

Mild 99%

Moderate 1% Sheep

Mild 98%

Moderate 2%

Alpacas

Mild 99%

Moderate 1%

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?

Research into influenza virus infection and associated complex virus-host interactions requires the use of biologically relevant animal species with a complete immune repertoire. Only viruses of scientific importance will be characterised by infection of the valid species of live animal. Viruses will be selected based on previously obtained clinical, epidemiological or laboratory data indicating that characterisation of parameters such as virus infectivity, pathogenesis and transmission is scientifically required using live animals. Additionally, the host response to infection may also be investigated. For example, the mechanisms of virus transmission between individual animals or disease interventions such as development of a protective immune response to influenza infection, cannot be studied in nonanimal alternatives.

The establishment is committed to use alternative technologies to replace antisera where possible using non-animal derived antibodies. However, in the specific case of influenza-specific hyperimmune serum generation, non-animal alternative *in vitro* technologies for veterinary species are currently do not exist and are not available. Furthermore, these technologies, even if they did exist for veterinary species, require mature splenocytes or B cells collected from animals that were previously immunised with the antigen in question. Additionally, influenza viruses are antigenically diverse and constantly evolving, so that universal antigens are lacking and standard reference antigens need constant Bioinformatic analysis and updating (every 6 months in the case of human seasonal vaccines). Scientifically valid hyperimmune sera need to authentically reflect the polyclonal

antibody response and breadth of humoral immunity elicited by influenza virus antigens composed of multiple proteins.

Which non-animal alternatives did you consider for use in this project?

In vitro and *ex vivo* methods, for example use of genomics, continuous cell lines and tissue explant cultures, are considered and frequently used to obtain virological data and allow preliminary scientific assessment of strains prior to animal studies being conducted. Additionally, less sentient forms, such as embryonated chicken eggs, are also used instead of sentient free living animals when appropriate.

However whilst they can replace aspects of the research the use of animal models is required to examine complex questions regarding host: pathogen interactions where a biologically relevant host is required.

Various animal free antibody techniques were also considered, either through literature search on discussion with companies supplying animal free antibodies. Whilst this technology is available for monoclonal antibodies currently there is no proven technology for the replacement of polyclonal antibodies which is required for the research undertaken using this licence. From this research it was considered animal free antibody technology may be able to produce multiclonal antibodies (a collection of monoclonals), rather than true polyclonals which are based on the genetic information on B cells of an existing animal producing the relevant polyclonal antibody or the antibody itself (phage display technology).

To this end under this licence, where appropriate blood samples are obtained from immunised animals, peripheral blood mononuclear cells, which contain mature B cells (Plasma cells) that produce antibodies having specific reactivity, will be cryopreserved should appropriate technologies become available in the future.

Why were they not suitable?

Alternatives to live animals are not suitable for the research of complex biological systems. Studies involving biologically relevant live animal species capable of mounting a complete immune response to infection are required to address scientific questions concerning the interaction of viruses with the host immune system during the infection process. The scientific aspects studied include investigation of progression of disease following virus infection (pathogenesis), virus transmission dynamics and virushost interactions as well as evaluation of the host response to infection eg generation of protective immunity.

Investigation and modelling of risk pathways in agricultural settings requires the use of populations of live animals under industry-relevant conditions e.g. assessment of swine influenza infection dynamics in a pig herd.

With respect to hyperimmune serum generation, Animal Free Antibody technologies developed to date only permit production of monoclonal mouse or human sera and there have been insurmountable hurdles in generating antisera in veterinary species aligned with the purpose of this licence. Furthermore, even if this technology became available for veterinary species, existing mouse hybridoma technology and phage display techniques require immunization of an animal to generate mature splenocytes or B cells, which are then harvested and require extensive screening to select the single clones amongst millions of cells that have the required reactivity. For the study of influenza, the development of an immune response to antigens in an animal species relevant for this licence with a complete immune repertoire is required in the case of influenza virus antigens to ensure the fidelity of hyperimmune serum reagents produced and cannot

currently be replicated using in vitro methods. Serum generated in non-veterinary species is not appropriate and will not achieve the required scientific purposes of this licence. In particular, antisera generated in an immunized animal is required to authentically reflect the polyclonal antibody response and breadth of humoral immunity elicited by influenza virus antigens from rapidly evolving influenza viruses composed of multiple proteins.

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 for this licence has been informed by studies completed under previous project licences and research programmes.

In the case of generating hyperimmune serum, a single animal will be used for each antigen. The number of antigens required during this licence will be informed by influenza surveillance activities followed by laboratory characterisation of viruses.

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, the animal groups and sizes required for a study are determined by the statistically valid minimum number of animals required to address the study hypothesis or aim. Animal study and group numbers are therefore designed in a consistent manner so that inter-study comparisons and statistically valid data analysis can be performed. This number is informed by studies completed under previous project licences and research programmes, established best practice in the influenza field and published research data. Expert advice is also received from a professional Biostatistician.

For the generation of hyperimmune serum, a single animal (the minimum) is used for each antigen.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Animal studies will be designed to maximise collection of biological materials and samples. When feasible, studies will be run concurrently with the objective of reducing the number of control groups required. These activities will aim to increase the data output and research questions that can be addressed in each study. Reagents arising from the use of animals, such as hyperimmune serum panels, will be shared between laboratories, to reduce the number of animals used by avoiding duplication of activities to generate the same hyperimmune serum reagent. Where possible, hyperimmune serum will be collected during the longitudinal monitoring or end of a study to reduce the number of animals used for hyperimmune serum generation.

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 natural livestock species that are the biologically and scientifically valid, natural hosts are used in this licence for influenza research. This license does not use other species to model infection of the natural hosts as these are not scientifically valid.

Embryonated chicken eggs which are less sentient embryonic forms compared to sentient (hatched) animals are used for virus isolation and propagation.

Prior to raising any hyperimmune serum, a case will be made using forms specifically for the production of antibodies using animals is justified. The AWERB reviews and assesses, each animal antibody production study form (ASUF 303 A) and annually the committee reviews antibodies produced in an assessment proforma (ASUF 841). These are attached in the action plan. If animals are used to produce hyperimmune antisera needs to be raised in the same species used for research work (namely, pigs, cattle, sheep and alpacas). Raising serum in other species, with genetically different immune systems, would lead to reductions in assay sensitivity and specificity or possibly produce scientifically invalid results.

When immunising animals, such as for raising of hyperimmune antisera or evaluation of immunogens such as vaccines and other disease-mitigating strategies, an inert immunogen will be used in preference to inoculation of infectious virus. Adjuvants, if required with an inert immunogen to ensure a sufficient immune response is elicited, will be non-ulcerative. These measures, avoiding the use of virus infection, are designed to cause the least possible harm while ensuring an effective immune response. The establishment has a lot of experience in raising antisera in other species and have techniques to minimise any adverse reactions to injections or blood samples that are needed.

Protocol 4, that use infectious virus to study pathogenesis, transmission and protective strategies, such as vaccination, has a severity level of moderate. A clinical scoring system is used to allow standardised clinical monitoring and define the signs associated with the mild and moderate severity level.

Instructions are provided as to the actions needed in response to individual or cumulative clinical score values. Increased scores of severity level from mild to moderate will result in an appropriate response such as communication with Named People (e.g. NVS, NACWO and Study lead PIL) and possible further responses such as treatment, including analgesic medication and/or increased frequency of monitoring.

Studies in agricultural settings are included to gain insight into influenza epidemiology in herds under field conditions and had a severity level of mild. Disturbance and sampling is minimised so the animals health and welfare as well as social environment are not disturbed.

Why can’t you use animals that are less sentient?

Where possible, less sentient forms are used. For example, embryonated chicken eggs that have been incubated for a shorter time than 48h before hatch and are defined as non-sentient in terms of Home Office severity recording. In studies involving investigation of

virus-host interactions, it is not possible to use less sentient animals as complete biological systems or animals with an intact immune repertoire are required to investigate the complex virus-host interactions that occur during infection.

The epidemiological investigations can only be done in the actual species that are affected.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Best practice procedures for animal husbandry, housing and environmental enrichment are implemented. Prior to study start, animals are acclimatised in their study groups and animal accommodation for a minimum of 7 days or longer if necessary. After acclimatisation, animal training and positive reinforcement are used during restraint and to obtain baseline samples and data. Pilot experiments are conducted to refine protocols e.g. dose, route and protocol of virus inoculation and to establish infection and transmission parameters.

Species- and disease-specific observation criteria and clinical score systems are utilised to ensure a standardized approach to clinical monitoring, limit duration of clinical signs and define humane endpoint criteria appropriate for the protocol. No animal is allowed to progress beyond the humane end point of the protocol severity level. Monitoring systems are reviewed regularly and updated when new information becomes available. On-site veterinary teams and Named animal welfare officers (qualified NVSs and NACWOs) participate in each study. Clinical signs serve as study endpoints when the scientific objective does not require progression of disease. Anaesthesia is used when administration is not more severe than the procedure itself. Appropriate treatment is allowed eg use of NSAID medication to treat fever.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Published information regarding best practise for relevant species is obtained from other research institutes, veterinary practitioners and organisations such as NC3Rs, IAT, LAVA/ LASA and the RSPCA. Scientific publications and articles are reviewed during the Ethical approval process prior to each individual study. Where specialist training is required, inter-institutional exchanges and training visits are organised. Best practise information is also shared within the influenza scientific community during conferences, meetings, inter-laboratory exchanges and through personal communication. The following guidance is reviewed regularly

HO Guidance to ASPA

ASPA Code of Practice

OIE (World Organisation for Animal Health) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.

LASA Guidelines on substance administration

NC3Rs web site

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

This Establishment is a signatory to Understanding Animal Research (UAR) and applies the Culture of Care in animal studies. The Establishment frequently attends or organises external symposia on laboratory animal welfare e.g. RSPCA and IAT meetings. Staff attending these meetings provide meeting feed back reports locally. The NC3Rs webpage (https://www.nc3rs.org.uk/) is regularly reviewed and a network of veterinary excellence supports all animal work at the organisation and provided updates regarding advances.

In addition, the establishment has a Species Group Care and Use Committee where all PILs are invited to attend. Specialist topics are presented and refinements, such as environmental enrichment, are communicated and implemented when appropriate. Additionally, specialist knowledge sharing in relation to specific protocols or species is organised through seminars, meetings and in-person exchanges with external organisations eg UKHSA, and other UK Government organisations, academia and international organisations such WOAH and the Worldwide Influenza Centre (WIC) at the Crick Institute, London.

90. Evolving increased vertebral counts: Developmental mechanisms underpinning phenotypic evolution in the Lake Malawi cichlid radiation.

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

phenotypic diversity, evolution, development, cichlid fish, vertebral counts

Animal types	Life stages
Lake Malawi Cichlids	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?

To understand how morphological diversity evolves in animals by studying the processes that guide their embryonic development.

In particular, we will address the question of how vertebral numbers evolve and use cichlid fishes from Lake Malawi as our model species.

There are many different species of Lake Malawi cichlids exhibiting vast diversity in many morphological traits, providing a unique opportunity to compare how this trait develops and evolves.

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?

One of the most fundamental and long-standing questions in biology is how morphological traits evolve to create the incredible diversity of shapes and forms seen in nature. Vertebrate body shapes provide a great example of this, ranging from the round ocean sunfish to the long, slender bodies of eels, with many variations in between. This diversity is largely due to the evolvability of their axial skeletons and the vertebrae that make them up. Vertebrae differ in size, shape, type, and—perhaps most remarkably —in number. The number of vertebrae can vary widely, from fewer than 10 in some frogs to several hundred in snakes, yet this number remains strikingly consistent within each species. This project will explore how vertebrate species evolve to have more vertebrae, aiming to uncover the developmental processes that drive this diversity and helping us better understand how species can evolve their morphology to adapt to new environments.

What outputs do you think you will see at the end of this project?

- We will identify the aspects of embryonic development that have led to the evolution of increased vertebral counts in some species of Lake Malawi cichlids but not in others
- Research publications in peer reviewed journals
- We will generate valuable resources for the research community such as genetically altered fish, gene expression datasets and live imaging of developmental processes.
- In the future, increased knowledge of how developmental processes evolve might be useful in bio-engineering applications such as organoid biology for regenerative medicine.

Who or what will benefit from these outputs, and how?

In the short term, the scientific community will benefit directly from these outputs via the publication of our research and data, but also via the sharing of fish models. In the long term we expect that other groups such as patients, clinicians and even the pharmaceutical industry might benefit as understanding development and how it can change can have far reaching application to regenerative medicine.

How will you look to maximise the outputs of this work?

- We will publish all our work open access
- We will make all our datasets and resources freely available upon publication
- We will communicate our results at conferences
- We will publish blog posts bringing our research to the broader public
- We will participate in science outreach events
- We will share our fish models and tissues with the scientific community

Species and numbers of animals expected to be used

- Lake Malawi Cichlids: 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.

Lake Malawi cichlids are a powerful emerging model system in evolutionary developmental biology. Around 850 cichlid species have evolved in Lake Malawi from a single common ancestor in under a million years. In this short amount of time, cichlids have colonised every available habitat in the lake and exhibit an astonishing degree of phenotypic diversity in traits such as body shape and size, colour and pattern, cranio-facial morphology, social and breeding behaviours, and finally, in vertebral counts, the trait that we will focus on. Despite this diversity, genetic variation in these fishes is unexpectedly low, with an average genomic sequence divergence between species of only 0.2% (a fifth of the divergence between human and chimpanzee, and similar to that found in human populations). The unique characteristics of the Lake Malawi cichlid radiation makes these species the ideal system in which to explore the role that developmental mechanisms play in generating phenotypic diversity. We will be using embryonic life stages, young, and adult fish.

Typically, what will be done to an animal used in your project?

This project will mostly focus on embryonic stages of development before free-feeding (when they become protected by the law). In some cases, embryos will be injected with genetic material, or exposed to cold or hot temperatures or a pressure shock to sterilise them. Larvae and late stage embryos will be injected to modify their reproductive cells.

We will generate genetically altered fish to follow how gene expression dynamics differ during development in species that differ in vertebral counts. These genetically altered fish will be bred using conventional methods.

Some animals will undergo clipping of their caudal or anal fin to correctly identify their genetic status.

Some animals will be humanely killed or, if they are not genetically altered, rehomed

What are the expected impacts and/or adverse effects for the animals during your project?

The genetically altered fish we will generate are expected to be perfectly healthy with no adverse effects predicted.

Techniques performed on adult fish: fin clipping for genotyping and generation and maintenance of genetically altered fish are standard techniques, with only transient discomfort and no lasting 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)?

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?

We need to use animal models to understand how differences in the embryonic development of closely related species can lead to the evolution of adult phenotypes, and with it, the evolution of new species and adaptations.

Whenever possible we work on fixed samples of early stage embryos, and many most of our experiments are conducted using this approach. However we cannot bypass the need to use embryos. In addition, no current datasets of these data exist that could be used instead of generating our own.

Which non-animal alternatives did you consider for use in this project?

I considered the gastruloid system which is a stem cell-derived 3D in vitro system which is used to model early mammalian development.

Mathematical models to simulate developmental processes.

Why were they not suitable?

3D gastruloid systems are unsuitable for this project because they don't produce the correct number of vertebral precursors for the species we study. We rely on these precursor numbers to determine the final count of vertebral counts.

We use data-driven mathematical models to simulate developmental processes, which cuts down on the use of animals but cannot replace them entirely as we need a whole organism to replicate whole body changes.

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 used estimates based on previous research using non genetically altered animals as well as published literature.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Cichlid fishes have complex social lives and we have found that tanks of around 50 individuals keep them at an optimal density that maximises their welfare by minimising the effects of territoriality and competition for mates. We will generate genetically altered fish at these densities to ensure we have access to enough embryos for 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 have optimised the breeding and husbandry of these fish to maximise their welfare, for example, our tanks have environmental enrichment including a sand substrate, plastic plants, plant pots, plastic tubes, and rocks. Lake Malawi cichlids benefit from such tank environments as this enrichment decreases harassment by providing hiding spot and more territories to be claimed for breeding.

We will ensure that embryos do not go to waste by either fixing and storing them for later use or dissection training for new staff. We will also feed excess wild type embryos at pre-free feeding (not protected) stages to our piscivorous species to complement their diets.

We use mathematical models of developmental processes instead of experimental approaches, to generate hypotheses which then target and reduce the need for 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 do most of our experiments on early stage embryos, pre-independent feeding and while the nervous system is still developing.

Adult fish will undergo fin clipping or skin swabs for genotyping. These procedures are very standard and used widely in labs all around the world, which means that they have been optimised already to reduce as much the pain and distress caused to the animals.

Why can’t you use animals that are less sentient?

Vertebrates are required to study the evolution of vertebral counts, which is the main aim of this project. Much of our work is done in early embryos but we still need to perform some of our protocols on the adults.

Less sentient species such as those either lacking a nervous system altogether like sea sponges, or those lacking a centralised nervous system such as sea urchins or jellyfish lack vertebrae and more generally a segmented body axis. Arthropods and annelids have segmented body axes but lack vertebrae.

Many of our data will come from analysing fixed (dead) embryonic tissue samples but we still need reproducing adults to gain access to embryos.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We always perform our procedure in the morning so that fish can be monitored during the day.

All our animals are regularly trained by expert fish technicians and lab staff.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Cichlid fish are still an emergent non model organism, which is why we heavily rely on the expertise of other fish labs to ensure best practice. We will however follow and adapt the guidance available for zebrafish where applicable. For example, NC3Rs provides excellent guidelines on how to assess pain and discomfort in fish.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly check relevant websites and resources such as www.nc3rs.org.uk and https://science.rspca.org.uk.

We will also regularly reach out to our network of international cichlid labs to see how they are doing these procedures and check whether anyone has come up with any innovations that might benefit our fish.

91. A mouse model of multiple myeloma

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, myeloma, NSD2, mouse, blood

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?

To develop and test a novel genetically altered mouse model for the cancer multiple myeloma.

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?

Multiple myeloma is a blood cancer arising from plasma cells. Around 1 in 100 people will develop multiple myeloma (MM) in their life time, equating to 6000 diagnoses in the UK every year. Despite advances in patient treatment over the last twenty years the disease remains essentially incurable and only 33% of patients are alive 10 years after their diagnosis. Understanding the genetic basis of how myeloma develops and evolves is crucial for rational drug development. Currently there are no mouse models of myeloma which adequately mimic the underlying disease biology. We have therefore developed a novel genetically altered mouse model which mimics MM biology in humans. Since development of myeloma cannot be modelled in cell lines and there are no animal models faithfully recapitulating myeloma, it is important to develop and characterise this model as

it will afford opportunities to better understand how the disease develops and test novel therapeutics.

What outputs do you think you will see at the end of this project?

The initial benefit of this project will be the creation of a genetically altered mouse model of multiple myeloma which faithfully recapitulates human disease biology. This system can then be used to both understand how the disease develops and test novel therapeutic agents to improve patient treatment. We will also make our model available to the wider scientific community for others to exploit.

We will analyse the genetic profiles of tumours potentially yielding insights into disease biology, these findings will be communicated in scientific publications and at meetings.

Who or what will benefit from these outputs, and how?

Improving our understanding of myeloma biology and the testing novel drugs has been restricted by absence of appropriate animal models. In the short term this genetic model system aims to provide insights into disease biology benefiting scientists. In the longer term we aim to provide benefit for myeloma patients by accelerating drug development.

How will you look to maximise the outputs of this work?

Our findings will be communicated via publication in scientific journals and presentations at scientific meetings.

We will deposit our genetic model into the national archive to make this available to the scientific community in conjunction with appropriate 3rd parties.

Species and numbers of animals expected to be used

- Mice: 1900

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 development of myeloma cannot be modelled in cell lines. It is necessary to use animals because they more closely mirror the normal biological environment in which cancer develops, for example including different cell types. Myeloma cells from patients cannot generally be grown or cultured longterm. Multiple myeloma tumour cells resides within the bone marrow, however the MM cell lines have been derived from circulating blood plasma cells or effusions at other sites. As they are derived from outside of the bone marrow, they do not reliably recapitulate the disease. As myeloma is a disease of plasma cells (a type of B-cell) we cannot use other commonly used animal models (e.g. zebrafish or flies). These animals don't possess the same antibodies that plasma cells produce and therefore are not analogous to mammalian species. Consequently, there are no viable alternatives to mice.

Our proposed programme of work will utilise other pre-existing genetically engineered murine models.

Procedures will be performed on adult mice as the disease is not expected to manifest in early life.

Typically, what will be done to an animal used in your project?

Our novel genetically modified mice will be bred with other pre-existing genetic models producing combinatorial models, each addressing an important question relating to myeloma development.

Since MM is predominantly a disease of older adults, animals with desired genetic modifications will be be immunized with a T- cell dependent antigen (Eight to 12 week old mice) to stimulate naïve b cell to differentiate to plasma cells and GC B cells and then monitored for signs of disease for up to 20 months. During this period every three months we will perform two procedures. Firstly, animals will have a blood sample taken. Secondly, animals will be anaesthetised and imaged to visualise tumour development.

What are the expected impacts and/or adverse effects for the animals during your project?

We anticipate that some animals will develop Monoclonal gammopathy of undetermined significance (MGUS) which usually is asymptomatic. Some of these animals showing MGUS will develop myeloma, leading to symptoms like mild anaemia, bone lesions and weight loss. Mice will be imaged at predefined timepoints for the presence of the disease and culled afterwards if the disease is confirmed or reached humane endpoint. These harmful phenotypes will only be experienced in a subset of the animals in the project. The genetic modifications being used are in an inactive state until they are

found in combination with an activating genetic change. We will restrict this from occurring until we are ready to study the disease. This will prevent animals in breeding colonies from experiencing harmfully effects due to genetic changes. The severity of harmful phenotypes will be limited by using defined humane endpoints, for which animal will be killed, when severity is reached.

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 animals developing cancer will experience a moderate level of severity.

We expect 100% of animals in the breeding protocol to experience a maximum of subthreshold severity.

We expect 50% of animals in the monitoring and imaging protocol to experience a moderate severity. The remainder we expect will experience a mild 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?

Multiple myeloma cannot be modelled in cell lines. Also, primary cells from patients cannot generally be grown or cultured long-term. As myeloma is a disease of plasma cells (a type of B-cell) we cannot use other commonly used animal models (e.g. zebrafish or flies). These animals don't possess the same antibodies that plasma cells produce and therefore are not analogous to mammalian species. It is necessary to use animals to provide the multi-cellular environment required to model these processes. Few 3D multiple myeloma and bone marrow organoid models have been generated, but they have certain challenges including short culture time, absence of various immune cells, stromal cells and osteoclasts which is are important constituents of bone marrow microenvironment etc. Furthermore, development of novel therapeutics requires animal models that faithfully recapitulate the biological process promoting human disease. A main project aim is to create and validate such a model system, consequently we must utilise animals.

Which non-animal alternatives did you consider for use in this project?

Human multiple myeloma cell lines and 3D multiple myeloma organoid models were considered as an alternative. However, the multiple myeloma cell lines were derived from outside of bone marrow and hence do not reliably recapitulate the disease. Organoid models have certain challenges including short culture time, absence of various immune cells, stromal cells and osteoclasts which is are important constituents of bone marrow microenvironment etc.

Why were they not suitable?

Cell lines do not completely recapitulate multiple myeloma as it lacks the presence of BM microenvironment. Primary cells from patients have short lifespan. Other commonly used animal models (e.g. zebrafish or flies) don't possess the same antibodies that plasma cells produce and therefore are not analogous to mammalian species. 3D MM and bone marrow organoid models have several challenges including shorter lifespan and absence of crucial immune cells, stromal cels and osteoclasts present in bone marrow microenvironment.

Drug testing can be performed in cells lines, however promising candidates must be validated in animals 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?

Statistical robustness is linked to disease frequency in test animals, the greater this frequency the lower the number of animals required to demonstrate an effect. We have calculated the number of animals required by estimating the number of animals we expect to develop disease in each arm of the experiment. Based on these estimates we have chosen the smallest number of individuals required to be sure there is difference in

disease incidence in different experimental arms. This is a new genetic model and full characterisation will be carried out in the initial studies in order to inform future work.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our experiment was modelled using the NC3Rs experimental design assistant. We have also had the design assessed by our in house statisticians and animal's welfare personnel within the Institute.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For the experimental breed, we have designed a breeding strategy which utilises 100% of the animals, helping to reduce the numbers euthanised due to genotype. In order to get to the experimental cross, there will be several breeding steps in order to generate the correct genotypes of the parents. We will use colony calculations based on known data about the line to estimate how many mice are needed at each stage to avoid producing excess animals.

We are collecting and archiving tissue from all test animals, while this increasing labour in this study, the measure will prevent the need to conduct follow up experiments for this purpose.

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 4 different strains of genetically altered mice, all strains are bred on the C57BL/6J inbred genetic background. We have used this model as it is necessary to perform our experiments in species producing the IgG subtype of antibody. Depending on the disease incidence of our current models, we may use other oncogenes as well.

We have developed a novel genetically altered mouse strain for the experiment. This strain will produce an excess of a protein, associated with the development of myeloma. Excess production from this gene and one other gene, produced in another genetically modified strain we are using, could have undesirable consequences, for example the development of cancer in a tissue other than the blood. We have therefore designed our experiment such that genetic alterations will only be experienced in plasma cells. This approach has two key benefits, firstly animals should only acquire myeloma and not another cancer and secondly when breeding we can prevent animals from unnecessarily developing disease. The combined effects of this refinement reduce the number of animals required and reduce the suffering of those remaining.

The mice expressing oncogenes related to MM development may experience moderate level of severity but the planned procedures will not cause anything other than mild and transient pain, suffering or distress to the animals. However to reduce pain and distress to

animals we will perform, where possible, our 3 monthly imaging and blood collection in one session under general anaesthetic.

We do anticipate that some of the animals will develop myeloma, consequently we will monitor various criteria like weight loss, anaemia, pain behaviours, piloerection, etc to detect any signs of distress. Animals will be monitored using imaging equipment in order to determine presence of disease, in most cases, prior symptoms developing.

Why can't you use animals that are less sentient?

Faithful modelling of myeloma requires a species produce IgG antibodies. Other commonly use animal models (e.g. Zebrafish or flies) do not have a complex immune system and do not produce plasma cells analogous to mammalian species. Consequently, there are no viable alternatives.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will implement new animal welfare best practice when made aware from sources such as the NC3Rs, LASA etc.

The main welfare implication of our study is the development of cancer in some animals. We will examine whether, animals developing cancer, could be euthanised earlier prior to humane endpoints criteria being met. Once the model has gone through initial validation, we are aiming to identify early indications of disease sufficient to satisfy the study objectives before the onset of discernible signs

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

NC3Rs, Arrive reporting guidelines, PREPARE guidelines, OBSERVE guidelines, LASA.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

With reference to the NC3Rs website, with whom I have registered and receive regular updates. I have also joined oncology network of NC3R. We will also discuss with colleagues and gather information from other organisations via NIO.

Suggested best practice changes, impacting our study, will be discussed with our NACWO and where relevant NVS to determine whether revisions to our protocols are necessary. In this event we will, submit an amendment to our licence protocol to reflect their adoption.

92. Injury mechanisms, trajectory and therapeutic targets for brain disorders with early-life origins

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 development, neurodevelopmental disorders, developmental origins of disease, age-specific therapy, 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?

This work aims to understand early life events that affect the brain and lead to neurological disease. The primary focus of this work is to establish the mechanisms by which injury occurs in the developing brain. The long-term consequences of early-life injury will also be investigated.

This work will assess the signalling systems that regulate normal development and may be altered by factors that increase risk of injury (i.e. genetic susceptibility or altered developmental environment, such as inflammation/infection). The overarching intention of this work is to identify age-specific regulatory mechanisms that could be targeted therapeutically to correct initial or subsequent injury, normalising the brain’s structure and function or reducing the severity of injury.

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?

Disorders of the brain that are diagnosed in childhood or adolescence, termed neurodevelopmental disorders, appear to be initiated during early brain development. Neurodevelopmental disorders affect ~4% of the population and represents a huge cost to society, in both in terms of financing medical treatment (for example, ADHD costs the NHS £3mil/year for newly diagnosed patients alone) and in societal terms. Early-life events, such as low birth weight or preterm birth (birth early than 37 weeks gestation), that disrupt early brain development, also increase an individuals' risk of later life brain disorders, such as stroke or dementia. We know that an individual's risk of brain injury (early or late) is affected by their genetics and sex, and that the severity, timing and frequency of events (e.g. infection, poor growth and nutrition, trauma) can alter brain development.

Brain injury that commences in development can change over time, as the initial changes in the brain interact with normal developmental processes and subsequent life events. These interactions may in themselves cause low-grade injury or reduce the resilience of the brain to subsequent injury. We believe that interventions, such as drug treatments, to reduce injury can be effective throughout the lifetime if they are correctly targeted to an appropriate age-specific developmental or injury process, and that early identification and treatment can reduce accumulative disease severity. Therefore, the aim of this work is to identify age-specific targets for therapy, as well as to understand the brain's own capacity to respond to and correct early injury. It will also determine the susceptibility to later life injury and explore systems for monitoring this risk. This work will improve our basic understanding of brain disorders. Our new discoveries will be directed to early identification of patients who would most benefit from treatment, and the determination of the type and timing of treatment most appropriate to patient needs.

What outputs do you think you will see at the end of this project?

The primary output expected at the end of this project is an advancement in our understanding of normal brain development, mechanisms by which this can be altered, and how these contribute to lifelong brain health. The findings of our studies will be shared with the scientific community through publications in peer-reviewed open access journals. Where we identify mechanisms of injury that might be relevant to human disease and could be useful for early diagnosis of disease and/or allocation of patients for particular treatments, we will work with collaborators to identify the appropriate assessment methods (e.g. blood test, magnetic resonance imaging (MRI)). Our findings will be shared through publications, conference presentations and discussions with clinical colleagues. Similarly, where this work has identified novel disease modifying drugs (most likely identifying new uses for existing drugs) we will share our findings through publications, conference presentations and work with clinical colleagues to advance our findings towards the clinic.

Who or what will benefit from these outputs, and how?

This a basic science project which will increase understanding of normal processes of brain development, mechanisms of injury and how these injury mechanisms vary with age (both in terms of initiation and the evolution of injury across development). The immediate beneficiaries of this project will be the scientific community with whom we will share our findings, improving the collective understanding of these important biological concepts.

As our research is focused on identifying processes of injury that a) define a point where treatment may be successful, and b) test potential therapies, we also expect in the intermediate term to benefit the clinical community. We will be utilising existing neuroimaging tools (such as MRI) and expect to provide insight to clinicians in their use to detect injury in patients and to allocate patients for treatment. We hope to develop new markers of disease that are more effective or efficient for detecting injury. We will also

benefit this community by testing existing drugs for new (age-specific) purposes, in addition to testing novel drugs that maybe identified through our work.

In the long-term, we will benefit patient communities with neurodevelopmental or neurodegenerative diseases, either directly through our work on markers of disease and treatments, or through the work of others in the field inspired by our findings. While long-term, this is an important benefit as neurodevelopmental disorders collectively affect approximately 4% of the population, with the burden of disease and care lasting throughout the lifetime. Neurodegenerative disorders exacerbated by earlylife events, likely affecting a further 5-10% of the population, and cause severe and distressing disease for patients and their families.

How will you look to maximise the outputs of this work?

We aim to maximise the clinical relevance of our work by combining assessments of brain changes detectable with MRI with assessment of behavioural changes. These techniques are used in clinical practice and can be compared to findings in our animal models, where we will also be able to assess the structural changes of the brain using standard pathological techniques. We are maintaining clinical collaborations and participate in many research forums in the London community to ensure that our work is relevant and appropriately focused to the needs of patients, and utilising relevant technological advancements.

Timely, open access publication is essential for sharing our work with the broader scientific community and is a useful step in ensuring additional collaborations to enhance our own work, as well as allowing other research groups to build on our findings. Similarly, we ensure that the research team is actively engaged in the relevant international societies and research organisation, and regularly attend conferences to present our findings and discuss the state-of-the-art with others in the community. Where necessary we will communicate unsuccessful approaches within publications and conference presentations.

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.

We will be using mice for these studies, with interventions or assessments being performed throughout the lifespan (foetal to adult). Mice have been chosen as we, as a scientific community, know a lot about normal mouse brain development and the injury processes that align with human disease. There are many existing, well-established models of human disease and disease mechanisms that we will be utilising, including models developed by our group and collaborators. These models have provided the basis of our current understanding of developmental brain injury and as we know these models replicate aspects of human disease, we can use them to test drugs that might reduce injury, or identify markers of disease that might be helpful in humans to improve diagnosis. We will also benefit from the existing mouse lines with unique and specific genetic modifications. The use of these genetically modified mice will allow more precise investigation of the role of specific cells, signalling molecules or disease relevant genes,

thereby increasing the scientific advances possible, while reducing the cost and overall animal use.

This project focuses on understanding developmental origins of disease, and it is therefore essential to make assessments throughout the lifespan. Mice have been proven to be useful for modelling aspects of human brain development and the subsequent ageing process. While not a perfect model of the human brain, they do show the processes of normal cellular development of the brain that are important for the present body of work. The use of model animals is essential, as we are aiming to understand the complex patterns of developmental and injury changes that occur in the brain over time, and the influence of changes in the body, outside of the brain (e.g. inflammation, poor nutrition), at different times during these processes. An intact body is required for this assessment of injury risk and accumulated injury burden across a lifetime, as well as for testing disease markers and treatments that be applied at different times after the initial injury. Adult animals will be required as a control for normal development, to compare between responses of younger and older animals and/or assess sensitivity to injury in later life that may occur as a result of early-life events.

Experiments are required that cannot be conducted in humans for ethical and scientific reasons, though where possible we will (through collaboration with clinical partners) confirm findings in patient samples (blood, brain tissue) and clinical imaging. We have considered the feasibility of achieving our purpose by not involving animals, for example by using cell lines in culture, but no such alternatives are able to reproduce the brain injury we aim to investigate in this proposal. Where possible we will perform screening in primary cell preparations or in appropriate cell lines prior to whole animal experiments. For example, these may be used to test the effects of altering disease relevant genes in a specific cell type or testing drugs to activate or inhibit specific signally pathways. We are currently utilising existing datasets, made accessible to the scientific community from clinical and animal experimentation, to test hypotheses without the need for new experiments, with those hypotheses supported by both these computational and cell culture methods prioritized for testing in our animal models.

Typically, what will be done to an animal used in your project?

Mice will be bred in social housing conditions and may be ear notched for identification purposes. When treatments to individual animals are required in the first two weeks of life, newborn pups will receive an injection of tattoo dye under the skin of the paw or tail. This technique allows individual treatment of pups in each litter with different injury or therapeutic agents, increasing statistical power and reducing overall animal use.

The majority of animals used in this project will be exposed to agents designed to alter how the body functions (e.g. inflammatory response, high fat diet) that will in turn affect brain development. These could include injections of a molecule that induces a mild inflammation multiple times over multiple days within the first week of life, or exposure to a diet designed to change metabolism (e.g. high fat high sugar diet) to assess how mild, clinically relevant early-life responses in the whole body affect brain development and sensitivity to injury in later life. In some studies, animals will also be treated with drugs which aim to reduce brain injury or improve behavioural abnormalities caused by the previous interventions. The effects of these challenges on brain structure or function will be assessed by a combination of tests to assess the animals behaviour or visualise the brain structures (e.g. MRI, post-mortem tissue sections). A typical animal may be exposed to 4 procedures across the course of a protocol, for instance i) neonatal tattooing for identification, ii) a perinatal challenge (diet, inflammation), iii) a postnatal therapy, iv) behavioural testing. Less typically, animals may be exposed to i) a perinatal challenge

(diet, inflammation), ii) a postnatal therapy, iii) a postnatal inflammatory challenge, iv) behavioural testing.

Many animals used in this study will have a genetic mutation (that will not affect the health of the animals) to facilitate assessments of the specific cells and molecules involved in the injury process.

What are the expected impacts and/or adverse effects for the animals during your project?

For animals receiving injections, we expect a transient period of discomfort at the site of injection (i.e. lasting less than 5min) related to the injection, with the possibility of an additional short-term period of discomfort (<24h) due to the agent injected (inflammatory mediator) or the solution it is dissolved in. When animals are due to receive multiple injections over consecutive days, the injection site will be altered slightly for each injection to avoid damage to the injection area. We have substantial experience with this method and this is typically well tolerated by the animals. Infection of the injection site is uncommon, but animals will be monitored for this and treated appropriately if infection should occur. Pups may stop eating during this period for a short time but are expected to continue to gain weight during this period, though they may do so at a slightly slower rate than littermates treated with saline.

Following administration of inflammatory agents, adult animals may exhibit sickness behaviours, e.g. piloerection, lethargy and huddling in bedding/corner of cage for up to 24 hours following administration. This is a sign of inflammation and leads to the desired changes to the brain. Animals may stop eating during this period for a short time but are not expected to lose a harmful amount of weight.

Animals receiving high fat high sugar diets are expected to gain weight and by the end of an 8-week exposure will have early signs of systemic metabolic changes (‘pre-diabetic’) on investigation but are not expected to show signs of altered health in their behaviour, or any signs of distress or discomfort.

Genetically modified animals may show mild signs of altered behaviour aligning with the disease of interest (e.g. repetitive behaviour for neurodevelopmental diseases), but these signs of disease will only be detectable on careful investigation and do not cause detectable distress or discomfort. In some cases, mice will be used with genetic modifications that may produce a slow injury to the brain over life. It is the aim of the work described here to study the early phases of disease, before any noticeable changes occur to the health of the animals. Studies which combine genetic modifications with earlier inflammatory or dietary interventions are expected to end before any clinical signs of diseases are detectable.

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)?

Overall, up to 40% of the animals used on this project are expected to have a moderate severity exposure across the course of their life, with the remaining 60% in the mild severity category.

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?

To achieve our aims, we need models that allow us to mimic human early brain injury in an environment where we can modulate the genetic and environmental causes of injury. We also need to be able to follow the consequences of the initial injury over a prolonged period of time. In particular, our experiments consider how multiple cell types interact over development and with injury, both within the brain and influenced by the responses that happen in the rest of the body. Experiments are required that cannot be conducted in humans for ethical and scientific reasons. In addition, for drug studies, it is necessary to assess how the drug moves around the whole body, and the timing in which it is able to have an action, which is dependent on organs such as the liver and kidneys. Animal models can achieve all these aims.

Which non-animal alternatives did you consider for use in this project?

We have considered the feasibility of achieving our purpose by not involving animals, for example by using cell lines in culture, but no such alternatives are able to reproduce the brain injury we aim to investigate in this proposal. We do, however, use these alternatives to refine our experimental approach and reduce the number of experiments performed in animals.

Why were they not suitable?

None of the existing non-animal approaches provide the capacity to study the interactions between different cell types and body organs, signalling mechanisms between cells/organs or the effect of time, in a manner that is sufficiently representative of the whole body situation to achieve the scientific aims of this 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?

Numbers of animals to be used have been estimated from a combination of data from previous work and use of experimental design tools. We have planned out likely future experiments and the number of animals required for these, should all planned work be funded and conducted by a full research team. The actual number of animals estimated for use here represents about a quarter of that number, which reflects the number of animals used under our previous project license and the likelihood that not all projects will be fully funded within the next 5 years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use the NC3Rs Experimental Design Assistant to design the experiments proposed and ensure that they will meet our scientific objectives, with the minimal use of animals capable of detecting statistically significant differences. We will use mice of both sexes in pilot studies, and where there are no discernible differences in the outcomes, data will be pooled to reduce the number of mice required. If the conditions of the pilot studies are unchanged, these data will be incorporated into the main study.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We reduce animal experiments by doing the first phases of hypothesis testing in existing datasets, online databases and using computer-based tools, as wells as in appropriate immortalized cell lines. This allows us to not only refine our hypothesis, but also to more accurately predict effect size and variation within experimental procedures, refining the design for animal experiments.

Because our work is done on postnatal animals, we are used to running a very careful breeding program designed to maintain the colony and provide the necessary animals for experimentation without producing greater numbers of animals than are needed. We will continue this practice for the current project.

Where possible, we ensure that tissue from a single mouse is used for multiple analyses. For example, we may have behavioural testing data, MRI and histological data from a single animal. This improves our capacity to make scientific deductions and reduces the overall number 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.

In this project, postnatal animals will be injected with agents (e.g. inflammatory mediators) designed to modulate the normal processes of brain development. Selected agents may cause acute sickness behaviour during the treatment period as they act on the brain and other body systems. Through this action they will subtly alter the development of the brain. These subtle brain injuries are only detectible with complex investigative methods and do not cause pain or distress to the animals outside of the administration period. Animals may also be treated with a potential therapeutic agent, designed to ameliorate the injury response.

When choosing how to administer these agents, we will always choose the least invasive method available to produce the required effect (that which most effectively mimics a mechanism of disease). We reduce the pain, suffering or distress during this administration by ensuring that we minimise time the pups are away from the dam, use the smallest needle calibre compatible with the injection volume and substance being

administered, and ensure staff are well trained in the procedures. If administration of an agent causes pain at the site of injection, a topical analgesic agent will be used.

We will also provide some animals with an altered diet (e.g. high fat high sugar) mimicking common human dietary variations that correlate with increase morbidity. Animals will have free access this food as part of their normal daily activities, clearly modelling human health in a manner that produces no overt stress or suffering. Diets will be time-limited, with animals returning to normal diets before any overt diet-induced phenotype (except weight gain) can occur.

Why can't you use animals that are less sentient?

We use immature, postnatal, mice for the majority of these experiments because their brain is at the appropriate developmental stages for the research questions being asked. We are studying the longterm effects of acute interventions on brain development and trying to identify potential drugs that can reduce injury. This requires a complete living animal that is able to recover from the initial intervention. It will also require occasional use of adult animals as controls for normal development, to compare postnatal and adult responses to stimuli, or assess response to multiple stimuli across a lifespan.

Species that are less sentient, while having many potential advantages, do not replicated mammalian brain development and inflammatory responses as well as rodents. We believe the use of rodents here, as a model for human developmental disease and subsequent risk of neurodegenerative injury, is important; we only use animals for experiments that cannot be done with alternative methods. In particular, less sentient species do not have a mammalian blood-brain barrier, and have a different vascular arrangement, which limits their suitability for studying vascular aspects of perinatal brain injury and the translation of findings to humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals on this protocol are monitored during procedures. As, in most experiments, postnatal animals are used and returned to mothers, monitoring has to be carefully balanced to allow assessment of the pups without disturbing the mother who may, if stressed, kill the litter in question. We monitor litters for rejection by the mother, lack of feeding (monitored through regular assessment of weight) and for normal age-appropriate behaviours. For the majority of the proposed experiments, animals may exhibit mild transient behavioural changes that last ~2-8 hours (<24h), representing minimal harm to the animal. If this sickness behaviour is substantial, or continues beyond the expected times, we will intervene through termination of the experiment, and, where necessary, enforcement of humane end points. In most cases, we are not able to ameliorate suffering with through use of typical analgesics (e.g. with NSAIDs) as they will alter neurodevelopment and the experimental paradigm. The exception to this is if animals receiving tattoo marking as neonates have a mild infection at the tattoo site, when local analgesics (e.g. lidocaine) and antiseptics will be used to reduce any pain and suffering caused by the infection. When using novel therapeutic agents, pilot studies will be conducted to confirm efficacy and to monitor for harmful side effects prior to use on a large number of animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

In addition to normal continuing professional development, supported by the RVC Biological Services Unit and Named Veterinary Surgeon, we actively follow NC3Rs

guidance regarding refinement of experimental procedures. We will also follow the published scientific literature in this area, supported by the RVC Biological Services Unit user group, to ensure that we are using the most up-to-date best practice methods in our field.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I am a member of the Biological Services Unit users group at the Royal Veterinary College. This forum presents new research and guidelines to its users and supports implementation of best practice in all experiments involving animals. I also participate in workshops and lectures organised by the NC3Rs and other research societies aimed at improving the reproducibility, scientific validity and refinement of animal experimentation.

93. Cellular & Molecular Mechanisms of Skin Development 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

Key words

skin, wound, scar, fibrosis, development

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 work is to understand how tissues repair after injury, with a particular emphasis on the mechanisms of scarring. The work proposed herein is on skin, where we are working understand anatomical variations in wound healing since different body sites vary in their mechanisms and quality of repair. We hope to ultimately extrapolate our findings from the mouse model to develop new treatments for human wound healing, fibrotic conditions, and site-specific skin 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?

There are many human skin diseases and wound-associated pathologies with insufficient treatment options. This work tests a novel hypothesis that the development of the skin (which differs depending on the body site) holds clues for the regional variation in wound repair, scarring and disease manifestation.

What outputs do you think you will see at the end of this project?

This project will lead to new information about: 1) skin development; 2) mechanisms of wound healing in the skin; and 3) regional manifestation of skin diseases. This information will be disseminated by publication as well as by conference presentations.

A potential longer-term output may include new treatment approaches to problems with wound healing, such as chronic wounds and excessive scarring, since this work aims to identify novel therapeutic targets.

Who or what will benefit from these outputs, and how?

Our short-term outputs (publications and conference presentations) will mostly benefit the relevant academic communities. We anticipate that our findings will have significant impact on scientific approaches to studying and modelling skin and wound healing.

If our longer-term goal of discovering novel treatment strategies to improve wound repair and limit scarring/fibrosis is realised, those who could benefit would be patients with wound-associated pathologies (from chronic wounds to keloid scars), and a range of skin diseases (e.g. lupus, scleroderma).

How will you look to maximise the outputs of this work?

The outputs of this work will be maximised namely by wide dissemination of the results (including negative findings) via publication and conference presentations. Moreover, we will continue to collaborate with researchers whose work would benefit from our alternative development biology perspective on tissue repair and associated pathologies.

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.

The relatively close evolutionary relationship between mouse and human make it an excellent model. Genetic resources in the mouse enable us to manipulate gene expression in different cell types and to genetically label and isolate distinct cell populations. Most of our studies will be conducted in young adults, however occasionally it

will be necessary for us to investigate tissue repair in neonates, at a time when the skin is still developing and the cells within the tissue are more flexible in terms of the type of cell that they will become.

Typically, what will be done to an animal used in your project?

In order to study skin wound repair, wounds are usually made to back skin of adult mice, and typically 4mm in diameter (4 may be made on one mouse) or 1cm in length (2 may be made on one mouse). Occasionally one 8mm wound will be made, or other sites of the body will be wounded to better reflect human diseases. For a small portion of our work, young pups (neonates) may be wounded since before a certain stage of skin development scarring is less severe and this provides a window of opportunity to better understand the mechanisms of tissue repair. These procedures are performed under anaesthesia with pain relief provided, are on average of a duration one week or less, and are considered of moderate severity.

All animals are humanely culled (Schedule 1) at the end of these experiments.

What are the expected impacts and/or adverse effects for the animals during your project?

The mice undergoing procedures on this licence are expected to experience mild or moderate effects, including transient (<48 hours) pain and stress due to the skin wounding protocol and treatments by injection. The genetic alteration to mice we will use are not expected to have any (or only mild) illeffects on health.

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)?

70% sub-threshold, 10% mild and 20% 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?

The complexity of tissue repair cannot be fully modelled in vitro or by using computer modelling because of the diverse cell populations and their intricate interactions. For example, recruitment of inflammatory cells from the circulation, or the importance of circulating hormones add layers of complexity that are impossible to fully recreate in culture or in silico systems.

Which non-animal alternatives did you consider for use in this project?

We are able to study certain aspects of wound repair ex vivo, with organ/tissue culture. Our team regularly employs culture experiments (using mouse and human tissue) to investigate how specific molecules affect particular aspects of wound repair. We have spent significant time over the last few years developing an in vitro model of extracellular matrix, which mimics the architecture of scar tissue well, and is amenable to manipulation. Ultimately we aim to extrapolate our findings to human wound repair; accordingly, we use human tissue in culture whenever possible.

Why were they not suitable?

Although some aspects of tissue repair can be studied in vitro or in humans, it is impossible to fully recreate scarring in a dish, and the heterogeneity of tissue damage and repair in humans is nearimpossible to experimentally "control".

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 maximum number of mice for this work is 10000.

This reflects housing up to twelve genetically altered lines (and the minimum number of animals required for their generation) for approximately 1 year of experimentation each. This will require up to 7000 mice.

For the wound experiments, we have performed power calculations based on our experience (6-8 wounds are required per assay per time-point) and have safely estimated the number of mice required to analyse two groups (e.g. control vs treatment/wild-type vs genetically-altered) at numerous timepoints. For the skin only, these numbers are then expanded since we will study multiple anatomical sites, at different stages of the life-course. These calculations have led us to predict usage not exceeding 1500 for skin wound studies.

Our phenotypic analyses of mouse strains that have novel timing/cell types for their genetic alterations will not exceed 1500 mice.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In designing these experiments we have referred to the Experimental Design Assistant (NC3R), and received statistical advice (in particular for gene expression profiling experiments). Also, as much as possible we use histological approaches since numerous wound parameters can be analysed with only one sample. Finally, we have consulted with a number of NVSs over the years to balance the size of the wound/number of wounds per

mouse, so that we can have the most statistically powerful data possible, as well as maximising the information gained per mouse, without causing additional distress (guided by important NC3R-mindful papers in the field: DOI: 10.1111/wrr.12148).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

One strategy we use to minimise animal numbers is, experiments are designed with a staged approach so that early in vitro findings rationalise and justify follow-up in vivo work. Also, to keep numbers at a minimum, animals used in the wound studies may also provide the starting material for some of the cell culture experiments. Finally, the numbers of genetically altered mice are minimised by adopting efficient breeding strategies (e.g. keeping homozygous mice when practical).

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 altered mice to study:

Mammalian skin development. The description of dermis development from different embryonic structures is poorly described; analysis of reporter strains over time is an essential addition to our understanding of skin biology.

Wound healing. The methodology has been refined such that the skin of adult mice (abdomen, back or cheek) is typically wounded using a biopsy punch that is only 2mm or 3mm in diameter. These wounds are as small as possible, yet sufficient to elicit a complete wound response and to provide the quantity of tissue required. All wounding procedures are performed under appropriate anaesthesia with analgesia. During and after all procedures mice will be carefully monitored. In our experience, wounded mice do not show signs of pain or suffering, eating and drinking normally.

Why can’t you use animals that are less sentient?

The generally similar skin development and architecture are what make the mouse the necessary model for this work.

At times we will study embryonic or neonatal mice, but as the skin is still developing, the response to wounding differs from adults.

Less sentient in vivo models of wound repair (e.g. zebrafish, Drosophila) are inappropriate because they don’t have an equivalent of a dermis that is susceptible to scarring.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will stay abreast of recommendations in terms of aseptic surgical technique, and analgesia. Heightened monitoring has the potential to minimise harm to the animals, and here are carefully monitored during the recovery from anaesthesia and again within 24h following surgery. Importantly, we will continue to minimise the wound size/number required for our scientific questions.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Home Office and ASRU documentation, as well as PREPARE and LASA guidance, and publications in relevant academic literature will be regularly consulted.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Via the NC3R charity, and through regular communication about best practice with our Biological Services Unit, the NVS and NACWO, and the users group.

94. Ecology and phenotypic impact of the rodent gut microbiome

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

microbiome, mice, gut bacteria, transmission, viruses

Animal types	Life stages
Wood mice (Apodemus sylvaticus)	Juvenile, Adult, Pregnant adult, Aged animal
Yellow-necked mice (Apodemus flavicollis)	Juvenile, Adult, Pregnant adult, Aged animal
Bank voles (Myodes glareolus)	Juvenile, Adult, Pregnant adult, Aged animal
Wild house mice (Mus musculus)	Juvenile, Adult, Pregnant adult, Aged animal
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 increase our fundamental understanding about what shapes the gut microbiome in mammals, and how gut microbiome variation affects animal physiology, immunity and behaviour.

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 recent years it has become clear that animals' microbiomes (the collection of microorganisms including bacteria, fungi, and viruses inside the body) can affect many aspects of their biology. The gut microbiome, in particular, can influence physiological, immunological, and even behavioural traits. This revelation has huge significance for developing strategies that maximise animal health - both in humans but also livestock and wildlife. However, much of our knowledge about how the gut microbiome affects host biology has been gained from laboratory animals (e.g. lab mice) which may not reflect what happens in the real world for several reasons - they have lost many of their natural gut microbes, have artificial microbial exposures, and greatly reduced capacity for natural behaviours. We therefore need to study wild animals and their gut microbiomes to understand how these microbes are acquired in natural settings, and what impact natural microbiome variation has on the animals' biology.

What outputs do you think you will see at the end of this project?

- Novel information on viruses found in wild UK rodent populations, including their zoonotic potential (those that can be transmitted from animals to humans) and how they generally spread, as well as information on how best to perform viral surveillance in wild rodents for potentially zoonotic agents, using modern sequencing-based approaches (metaviromics).

- Novel information about what causes differences in the gut microbiome of wild mammals, including which genes may be important in shaping microbiome variation, and how environmental and social factors play a role.

- New insights into the mammalian gut microbiome and how it affects animal physiology and behaviour, both in natural populations and laboratory mice.

- A set of wild-derived but pathogen-free mouse gut microbiomes that will be made available to other researchers, as well as associated phenotypic data on how these microbiomes alter various phenotypes compared to the lab mouse microbiome.

- Peer-reviewed publications on the investigated topics and outcomes made available to other researchers but also the general public via open access, and public engagement.

Who or what will benefit from these outputs, and how?

Within the timeframe of this project, the scientific community will benefit from new knowledge about gut-dwelling infectious agents in wild rodents, including viruses, bacteria and fungi, what shapes their epidemiology and what impact they have on their host's physiology and behaviour.

Also in the short term, researchers using lab mice as a model organism will benefit from the availability of new wild-derived but pathogen-free mouse microbiomes that can be used to understand a variety of biological processes or diseases in a more realistic microbial context. In the medium term, such 'rewilded' mouse models could also help the research community and pharmaceutical industry produce therapies (e.g. drugs and vaccines) that more accurately mimic human responses, thus improving the translation of preclinical research to clinical trials, ultimately benefiting the wider public.

In the longer term, the wider public will also benefit from findings on rodent viruses that inform how best to perform virus surveillance of wildlife, in order to monitor potential zoonotic threats and reduce the risk of these viruses spreading to humans.

How will you look to maximise the outputs of this work?

We routinely publish open access peer-reviewed papers and make the associated data (and typically also code) publicly available either in the paper or through linked data repositories and will continue to do so in this project. We will present work at scientific meetings, typically prior to publication.

We collaborate with multiple other groups throughout the UK and internationally. I recently co-founded a local network for microbiome researchers with regular meetings and workshops to facilitate collaboration and highlight resources available to share. I will continue to actively engage in this network throughout this project, facilitating sharing of knowledge and tissues from this project. Several immunology groups are already starting to use tissues derived from the 'wild gnotobiotic' mice generated on our last project (those inoculated with wild mouse microbiomes), and there is growing interest in this area. We aim to capitalise on this and provide access to tissues, wild microbiomes (faecal matter) and gnotobiotic mice from this project with other researchers, as this project develops.

My lab has also previously developed new less invasive radio frequency identification (RFID) based tools for monitoring wild rodents, and disseminated these through networking and talks/posters at conferences and NC3Rs events. They are now successfully in use by multiple other groups in the UK, Europe and the US. We will continue to use this technology and disseminate it as part of this project.

Our group also has a good track record in public engagement via a BBC wildlife programme, in online videos explaining our research, magazines for school-age children, news articles, and we regularly take part in public-facing talks and events to share our work with the wider public, as well as social media when appropriate. We will continue such outreach work as part of this project to maximise its impact.

Species and numbers of animals expected to be used

Mice: 2500

Other rodents:

Wood mice (Apodemus sylvaticus): 1000

Yellow-necked mice (Apodemus flavicollis): 200

Bank voles (Myodes glareolus): 400

Wild house mice (Mus musculus): 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.

This project will involve studying wild rodents (mice and voles, with a focus on two common mouse species), as well as laboratory mice.

For parts of this project on viral epidemiology and designing optimal surveillance approaches for potentially zoonotic viruses we will study natural populations of UK woodland rodents. We have chosen these species as they are ubiquitous in Europe, highly amenable to trapping and monitoring their spatial ecology in detail, have relatively stable populations suitable for long-term study and there is a wealth of pre-existing information about their ecology and infections that we can usefully draw on. They also frequently come into contact with humans, yet unlike house mice their viral diversity is less wellcharacterised, making them promising candidates for novel virus discovery.

To understand the causes and consequences of natural gut microbiome variation, wild house mice represent an ideal model system. This is because using this species, unlike others, we can powerfully bridge from field studies to the laboratory. In particular, we can perform microbiome transplant experiments from wild into germ-free animals in the lab, to understand the consequences of natural microbiome variation. Germ-free house mice are the gold standard for microbiome transplant experiments as they allow the cleanest manipulations. Furthermore, there are also a wealth of tools and knowledge available for this species that we can apply in either our lab or field studies.

Our field studies will involve capture and study of animals ranging from juvenile to adult (all those that can freely enter traps). By collecting data from these life stages we can get a full picture of viral and bacterial epidemiology, and how the microbiome changes with age.

In the laboratory, studies will mainly involve adult mice. However for some objectives (when the microbiome's influence occurs during early during development) we will need to either introduce bacteria to pregnant females and then study their offspring, who will receive their mother’s bacteria at birth, or raise mice with a defined microbiome.

Typically, what will be done to an animal used in your project?

In field studies we will catch wild rodents (mice and voles), assess basic characteristics such as species and sex, and measure morphological features. In some cases, we may collect ectoparasites (fleas, ticks) from their fur in order to assess transmission patterns of these organisms. Animals will typically be implanted with a small PIT (passive integrated transponder) tag under the skin (without anaesthesia) to permanently identify them and allow us to subsequently monitor their natural movements upon release using radio frequency (RFID) devices in the field. We will usually take a small notch from the mice's ears to get a tissue sample for genetic testing. Some mice (<10%) will be subjected to a short behavioural assay to assess locomotor, exploratory or social behaviour. A subset of wild-caught mice (<10%) will also undergo assays to measure their metabolic activity using indirect calorimetry and/or a thermal challenge, where they are exposed to a cold temperature for up to 2 hours, while their body temperature response is monitored using either using a previously subcutaneously implanted tag or thermal imaging. Some mice may also be given an oral anti-parasitic drug treatment to remove helminth parasites (<5%), in order to experimentally test how such infections affect the bacterial gut microbiome. Faeces will be collected from mice non-invasively to characterise gut-dwelling organisms and the gut microbiome, and some animals (~5%) may be blood-sampled to characterise blood-borne viruses. The vast majority of animals will be released at their point-of capture on the same day. A proportion of wild-caught mice (<10%) may be humanely killed rather than released, allowing us to measure internal characteristics of interest (internal parasite burdens after an anti-parasite treatment experiment, gut characteristics and fat deposit sizes). Mice will only be culled to measure internal

characteristics when necessary, for example when non-invasive proxies are insufficient. For example, we have found that in wild mice externally measured body condition indices do not correlate well with internal fat deposit sizes, and for some parasites internal burdens are not accurately reflected in faecal egg counts.

In laboratory studies, we will first produce mice with a specific microbiome (gnotobiotic mice) by inoculating juvenile or adult germ-free animals (those with no microorganisms) with either a microbiome derived from a wild or lab mouse, or non-pathogenic cultured gut bacteria. In order to understand the microbiome's influence in early postnatal development, some animals will be inoculated mid-pregnancy so that their offspring are naturally colonised at birth. We will also breed gnotobiotic mice. Germ-free as well as conventional lab mice may be maintained for short time periods (up to 5 months of age) to provide bacteria-free or conventionally reared control animals.

We will then characterise gnotobiotic and control mice to test the impact of different microbiomes on metabolism, physiology and behaviour. To do this, some animals will be given alternate diets in order to understand how the microbiome supports nutrient extraction from different food sources. Some mice may also undergo food restriction for up to 6 weeks, to test how natural microbiomes allow nutrient extraction under conditions of high or low food availability, as would be experienced in wild settings.

To understand the microbiome's impact on metabolism and thermoregulation, some animals will be implanted subcutaneously with a small device to measure body temperature, and undergo either a short-term thermal challenge (exposure to warm or cold temperature for up to 6 hours) or longer-term acclimation to a warm or cold temperature, by housing at different temperatures for up to 40 days. Some mice will undergo an assay that measures non-shivering heat production through brown fat, involving injection of a hormone and monitoring the metabolic response. Some mice will undergo indirect calorimetry in a home-cage environment, where O2 consumption and CO2 production are monitored from mice housed individually for up to 30 days, to measure metabolic traits like energy expenditure. To measure an animal's propensity to enter torpor (a state of reduced metabolism that some mammals use to save energy), some mice may be subjected to short-term food restriction in combination with housing at a slightly cool temperature (19-22oC) while simultaneously measuring body temperature through an implanted device. Some mice may undergo an assay to measure their metabolic response to glucose or insulin through standardised assays involving periodic blood sampling after injection of these substances. Blood sampling may be performed on some animals to monitor metabolic hormones or bacterial metabolites under particular conditions. Some mice will be subjected to non-aversive behavioural tests that assess locomotion, exploratory behaviour/anxiety or social behaviour.

What are the expected impacts and/or adverse effects for the animals during your project?

In field studies, wild-caught rodents inevitably experience some stress as a result of capture, handling and experience of novel environments while in captivity. Ear notching and tagging are expected to cause momentary pain. Mice given a cold challenge are expected to reduce movement and shiver, with a small risk of hypothermia or signs of pain/discomfort from this assay (<2%) that are reversible upon rewarming.

In laboratory studies, microbiome inoculation of germ-free mice may cause loose stools for several days that then resolves.

Altered diets and food restriction may induce either weight loss or mild weight gain. Mice exposed to a short-term cold challenge are expected to show reduced movement and shivering for up to 6 hours, with resumption of normal behaviour upon rewarming. Mice that are allowed to acclimate to a cold temperature may also show similar signs for several days prior to physiological accommodation. Mice under food restriction may lose weight. Mice administered insulin may develop transient hypoglycaemia and appear lethargic, which is resolved by administration of a glucose solution.

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)?

Wood mice: Mild 95%, Moderate 5%

Bank voles: Mild 98%, Moderate 2%

Yellow-necked mice: Mild 98%, Moderate 2%

Wild house mice: Mild 98%, Moderate 2%

Mice: Mild 45%, Moderate 16%, Sub-threshold 46%

What will happen to animals used in this project?

- Killed
- Set free
- 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 our field studies, a key goal is to understand how interactions between mice and their natural environment influence the communities of symbiotic microorganisms they host, and in turn how these communities shape affect their biology. As such it is essential to capture and sample live animals in their natural habitat, and gather data and samples of their natural microbiomes. Wherever possible, we will use assays on samples obtained passively from animals (e.g. faecal samples), rather than performing procedures to obtain samples or acquire information. To understand the phenotypic consequences of microbiomes on behaviour or complex physiology, no alternatives to animal use are available.

For our lab studies, mice are the best models to use as germ-free and gnotobiotic mice are available and allow us to powerfully examine how host host-microbiome interactions impact whole organism biology – physiology and behaviour. Such traits cannot be measured without animal use.

Which non-animal alternatives did you consider for use in this project?

We have considered the use of in vitro models of host-microbiota interaction (e.g. gut-on-a-chip – a device that can simulate the gut to explore how it interacts with gut bacteria) to understand how alternate microbiomes influence host tissue responses, and mathematical models.

Mathematical models will be used to model viral epidemiology in wild rodent populations, but these will require real-world data for validation.

Why were they not suitable?

Gut-on-a-chip models can measure how host gut tissue responds to a particular microbial community. However, physiological effects of the microbiome frequently occur through effects of gut microbes and molecules on cells and organs beyond the gut and a complex network of interactions involving both host and microbe components, which cannot be captured by such in vitro models. These in vitro models are also not suitable to infer the impact of alternate microbiomes on whole organism phenotypes like thermoregulation, metabolism and behaviour, which are key focal traits in this project. If particular molecular signatures of such physiological responses are detected in the gut through this work, in vitro models may become more useful approaches in future 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?

Animal number estimates have been based on previous experience of capture rates per species over the past 4-8 years at our main study sites (for wild rodents), and on typical experiment sizes and planned work using laboratory mice.

The numbers of animals in the laboratory have been based on previous projects and the literature to minimise the numbers of animals bred and then phenotyped to achieve the scientific outcomes.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All field experiments will be controlled and replicated, with randomisation to treatment groups and experimenter blinding at as many stages as feasible. Experimental design and power calculations will be informed by our prior experience of similar field experiments, and that of our collaborators. As wild animal capture rates can be highly variable, and effect sizes are less predictable than in a laboratory setting, the number of animals used in field studies cannot be firmly predetermined. Rather, using prior experience of capture rates and population density at our study sites, together with relevant estimates of effect size, we will design experiments to ensure an adequate, but not excessive, sample size is used. For longitudinal studies, sampling designs will be designed so as not to induce bias.

Laboratory experiments will be designed using good experimental design principles, including the use of power calculations with effect size estimates from prior relevant studies, use of appropriate control groups, randomisation (and blocking where

appropriate), strategies to control for confounders, standardisation of methods (e.g. sampling times) and husbandry techniques (e.g. cage changes) to minimise experimental noise, and blinding of researchers to treatment groups/donor phenotypes, both during laboratory studies and analysis of the resulting data, as far as possible.

Freely available tools such as the NC3Rs Experimental Design Assistant, as well as our own statistical experience will be used to design experiments that use the fewest possible mice to achieve our desired goals. To maximise reproducibility (and promote Reduction beyond this project), we will ensure published data are made freely available, interpretable by others and therefore useable in systematic reviews and meta-analyses, by following the NC3Rs ARRIVE guidelines and GRAFT guidelines for reporting mouse faecal microbiome transplant studies. Our experimental designs will follow several key principles arising from best practices to maximise power in gnotobiotic experiments this including standardising husbandry practice and sampling times, not pooling donor samples, and minimising the number of mice per cage as much as possible to maximise statistical power, while also maximising welfare.

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 maintain careful oversight of gnotobiotic mouse lines to ensure only sufficient animals for experiments and line maintenance are produced. We will store faeces for future re-inoculation rather than maintaining gnotobiotic lines that are not in active use (equivalent to cryopreservation of genetic lines). Where data is not available in the literature to determine appropriate sample sizes, we will use pilot studies. We have regular lab meetings to discuss planned and ongoing experiments, so we can foresee any possibilities to share animals across objectives and optimise tissue harvesting. At the end of an experiment, we will harvest as many tissues as possible, 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.

This project will use mice in both the wild and the lab, as well as wild voles.

Given our focus on the mammalian gut microbiome and interest in thermoregulation, mice are the species of lowest neurophysiological sensitivity that are appropriate for this research. As the dominant species in mammalian laboratory research we can draw on a wealth of knowledge about their biology and welfare to strive for the most refined experience possible.

Field study methods primarily involve capture-mark-recapture (trapping-based) studies, involving marking animals and collecting faecal and blood samples to characterise their microbiomes and other infectious agents. Captured mice may also undergo short assays to measure behaviour, metabolic rate (using indirect calorimetry) and response to cold

temperature. Some mice may be involved in an antiparasite treatment experiment where the least invasive suitable delivery method will be chosen. If anything, parasite removal is expected to increase rather than decrease health.

In laboratory experiments, we will generate gnotobiotic mice by inoculating germ-free mice with a known microbiome, before measuring various phenotypes either in inoculated mice or their progeny. Phenotyping may involve restricting food or altering diet to measure the microbiome's impact on nutrient extraction, or to induce torpor. We will use the minimum extent of food restriction required to generate the desired response, with pilot studies used to determine this where needed, starting with small numbers of animals subjected to mild restriction or diet alteration/supplementation.

We have already optimised the duration of cold challenge assays to the minimum required to observe the expected physiological response, and regular monitoring throughout ensures animals do not suffer lasting harm. Typically we use subcutaneous telemetry implants to monitor thermal responses as these constitute a more refined approach than many other methods (including surgery to implant a telemetry device for monitoring core body temperature or use of a rectal probe), and allow continuous monitoring of body temperature in case of an adverse response (hypothermia). Indirect calorimetry will be

performed in a home cage environment with enrichment, for the minimum time required to collect required data. Glucose and insulin tolerance tests, and blood sampling will follow highly standardised methods that are as refined as possible.

Why can’t you use animals that are less sentient?

A key goal of this project is to understand how well knowledge about the causes and consequences of gut microbiome variation derived from laboratory mouse studies, translates to natural settings. This goal requires field studies on mice specifically, as well as an ability to bridge between field studies of a model organism and controlled experiments to test how naturally occurring microbiome variation influences phenotypes like endothermic thermoregulation. These goals cannot be tackled in less sentient species such as fish or flies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our procedures for wild rodents have been refined over the years to maximise welfare, for example by setting limits on suitable weather conditions for trapping, using sensitive handling methods that minimise stress, transporting animals with minimal disturbance, providing ample food and water (or hydrogel) for mice that are kept for longer periods, careful study design and efficient planning so that animals can be released to the wild as quickly as possible. Wild-caught mice will be closely monitored during cold challenge (using subcutaneous temperature-monitoring implants and/or regular visual checks and thermal imaging) with clear trigger points in place to ensure timely intervention and recuperation as needed, as well as recovery prior to release.

All laboratory mice have at least daily health checks as standard and non-aversive handling techniques are utilised wherever possible, such as cupping and tunnelling (where animals are moved using a cup or tunnel rather than the tail, reducing stress). When mice will undergo regular handling or procedures, we will also try to habituate them to handling using a progressive approach across handling occasions as recommended by the 3Hs initiative. Mice are only singly housed when necessary for scientific or welfare reasons (e.g. during indirect calorimetry, or if required due to fighting). Following NC3Rs guidelines

for reducing aggression, we will typically use a mouse strain that is less aggressive (C57/BL6), co-house mice either with littermates or only mix litters at weaning to avoid male fighting, and if fighting is detected, separate those individuals. Enrichment is provided as standard and extra enrichment is provided, where possible, if animals are single housed. Microbiome inoculation of pregnant females will be performed as early as possible in pregnancy to minimise stress. In the days after microbiome inoculation, animals will be carefully monitored for changes in weight, body condition, or dehydration.

During procedures where weight loss is expected, body weight will be regularly monitored and carefully recorded allowing us to intervene as needed, for example with more frequent monitoring, or recuperation when necessary by providing wet mash. If a previously untested dietary supplement is to be used, pilot studies will be performed starting with low doses to identify a suitable degree of food supplementation that achieves scientific objectives in the most refined way.

During cold exposure of lab mice we will continuously monitor animal activity (using infra-red technology) and body temperature such that we can rapidly intervene if mice shown signs of inactivity or hypothermia.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will plan and report experiments in accordance with the PREPARE (Planning Research and

Experimental Procedures on Animals: Recommendations for Excellence) and ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines respectively, and consult relevant guidance published by the Laboratory Animal Science Association (LASA) and the NC3Rs (e.g. 'Breeding and colony management', 'Handling and training of mice and rats for low stress procedures' and 'Housing and husbandry: mice').

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have signed up to the NC3Rs newsletter and attend regular internal 3Rs meetings to keep ourselves updated on best practices. My lab has previously developed less-invasive methods for monitoring wild rodents and continue to actively search for and consider less invasive ways to conduct our research.

95. The Movement and Connectivity of Fish Populations

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
  - Improvement of the welfare of animals or of the production conditions for animalsreared for agricultural purposes
- Protection of the natural environment in the interests of the health or welfare of man oranimals
- Research aimed at preserving the species of animal subjected to regulated procedures aspart of the programme of work

Key words

Movement, Connectivity, Fish, Biology, Ecology

Animal types	Life stages
Gadoids	Juvenile, Adult, Pregnant adult, Aged animal
Clupeiformes	Juvenile, Adult, Pregnant adult, Aged animal
Rajidae	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult, Aged animal
Carcharhiniformes	Neonate, Juvenile, Adult, Pregnant adult, Aged animal
Lamniformes	Neonate, Juvenile, Adult, Pregnant adult, Aged animal
Scombriformes	Juvenile, Adult, Pregnant adult, Aged animal
Hexanchiformes	Neonate, Juvenile, Adult, Pregnant adult, Aged animal
Squaliformes	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?

To improve our knowledge of the movement of Osteichthyes (bony fish) and Chondricthyes (cartilaginous fish - sharks, rays, and skates), hereafter “fish”, including the drivers of movement, management implications, and incorporating fish movement in marine spatial planning.

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?

Fish face increasing pressures, which are detrimental to their populations. Direct pressures, such as overfishing, can directly affect population numbers, movements, and life history characteristics. In contrast, other pressures, such as pollution, development, and anthropogenic-driven climate change, have the potential to change the habitats fish rely on for their survival, forcing population changes such as distributional shifts and rapid evolutionary adaptations. These pressures have driven several fishes to the brink of extinction, with one sub-class, the elasmobranchs, numbered amongst the most threatened vertebrates on the planet. This highlights the urgent need for data collection to help mitigate these pressures and protect these species from further harm.

Studying fish movements and their drivers helps us understand behaviours, migration patterns, and habitat preferences. Combining movement knowledge with insights into species-specific biology, such as reproductive strategies and life history traits, can significantly increase our ability to identify critical habitats that should be incorporated into effective management strategies and targeted conservation measures to ensure sustainable fish populations. Beyond species-specific benefits, understanding the ecology of a species provides valuable insights into their role within their ecosystems and the broader environment. Fish play integral roles in nutrient cycling, predator-prey dynamics, and ecosystem stability. Thus, changes in fish populations can have cascading effects on entire ecosystems and, for more mobile species, multiple connected systems.

Each species in this project plays an important ecological role in marine ecosystems. They are also highly connected through predator-prey and niche competition interactions. For example, elasmobranchs (sharks, skates, and rays) and tuna feed on mackerel, gadoids (e.g., cod and haddock), and clupeiformes (e.g., herring), often with competition between the predatory species. Due to these complex connections, impacts on the movement, distribution, and population health of one species can have a much wider impact on other species. Therefore, it is crucial to understand the levels of interaction between these species across the different trophic levels. Research into these interactions is vital for developing effective conservation strategies and sustainable management practices. By understanding the intricate interactions between these species, we can better protect them and maintain the health of marine ecosystems.

Providing data on the movement, biology, and ecology of all these species to integrate this knowledge into conservation planning and management strategies can significantly

contribute towards conserving fish populations and support a move towards more dynamic ecosystem-based conservation management that has multiple benefits:

Enhanced Fish Population Conservation: By understanding species-specific behaviours, migration patterns, and ecological requirements, strategies can be tailored to mitigate threats and ensure sustainable fish populations.

Dynamic Ecosystem-Based Management: Incorporating ecological data allows for the development of adaptable conservation frameworks that address the complexities of marine ecosystems, fostering resilience to environmental changes and human impacts.

Multi-Stakeholder Benefits:

Commercial and Recreational Fishers: Improved fish stock management leads to more predictable and sustainable yields, supporting economic stability and recreational enjoyment.

Offshore Energy Providers: Data-informed strategies reduce conflicts between energy infrastructure and marine species, promoting coexistence and compliance with environmental regulations.

Managers: Access to comprehensive ecological data enhances decision-making, ensuring policies are scientifically robust and effective.

Aquaculture Industry: Understanding the natural ecology of species informs sustainable practices, reducing risks like disease transmission and habitat degradation.

Ocean Health and Biodiversity: Ecosystem-based approaches maintain ecological balance, benefiting not just target species but entire marine communities, thereby enhancing biodiversity and ecosystem services.

Climate Change Mitigation: Species-specific data help predict and mitigate the effects of climate change, such as shifting habitats and altered food webs, enabling proactive management measures.

Policy and International Collaboration: Sharing and utilising this data fosters alignment among conservation policies and international efforts which is essential for managing migratory and transboundary species.

What outputs do you think you will see at the end of this project?

The expected outputs of this project are:

Peer-reviewed publications.

Publicly available reports and data sets.

Conference presentations.

Tissue sample and image library.

Public outreach presentations.

Data to increase species knowledge and underpin policy.

Improvements in animal welfare through the refinement of research methods.

Who or what will benefit from these outputs, and how?

It is envisaged the outputs will be of benefit to multiple groups:

Researchers and students at this establishment via procedure training.

Government agencies will be able to use the outputs to support the development of conservation management.

Other research groups, both at this establishment and others, via the provision of samples.

Students, both at this establishment and others, via the provision of data and samples.

Stakeholders (e.g., commercial fishers and offshore developers) through data generation to support more refined and adaptive management of aquatic resources.

Wider public via increased awareness of fishes.

Animal welfare improvements via the refinement of research methods.

How will you look to maximise the outputs of this work?

The outputs from this project will be made as accessible as possible.

Publish in open-access journals.

Make data sets open access (upon publication).

Present outputs and any advancements of the 3R's at scientific conferences and workshops.

Send outputs to policymakers and special advisory groups.

Present at stakeholder workshops.

Make samples available to other researchers to support 'Reduction'.

Make protocols (SOPs and DOPs) available to other researchers either directly, or via published Open Access manuscripts.

Species and numbers of animals expected to be used

Other fish:

Rajidae: 1300

Carcharhiniformes: 700

Lamniformes: 700

Hexanchiformes: 700

Squaliformes: 700

Clupeiformes: 200

Gadoids: 200

Scombriformes: 500

Predicted harms

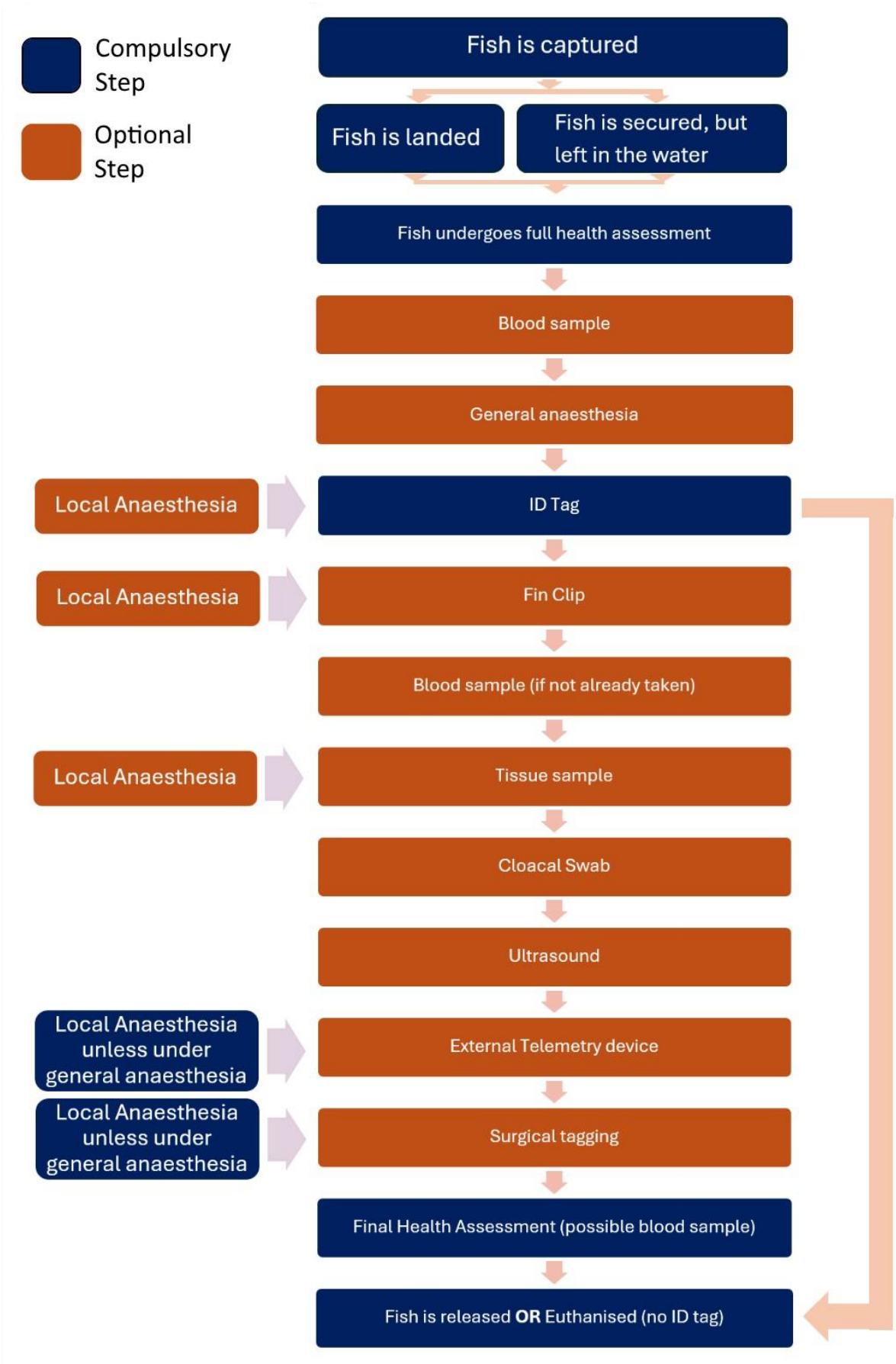
Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

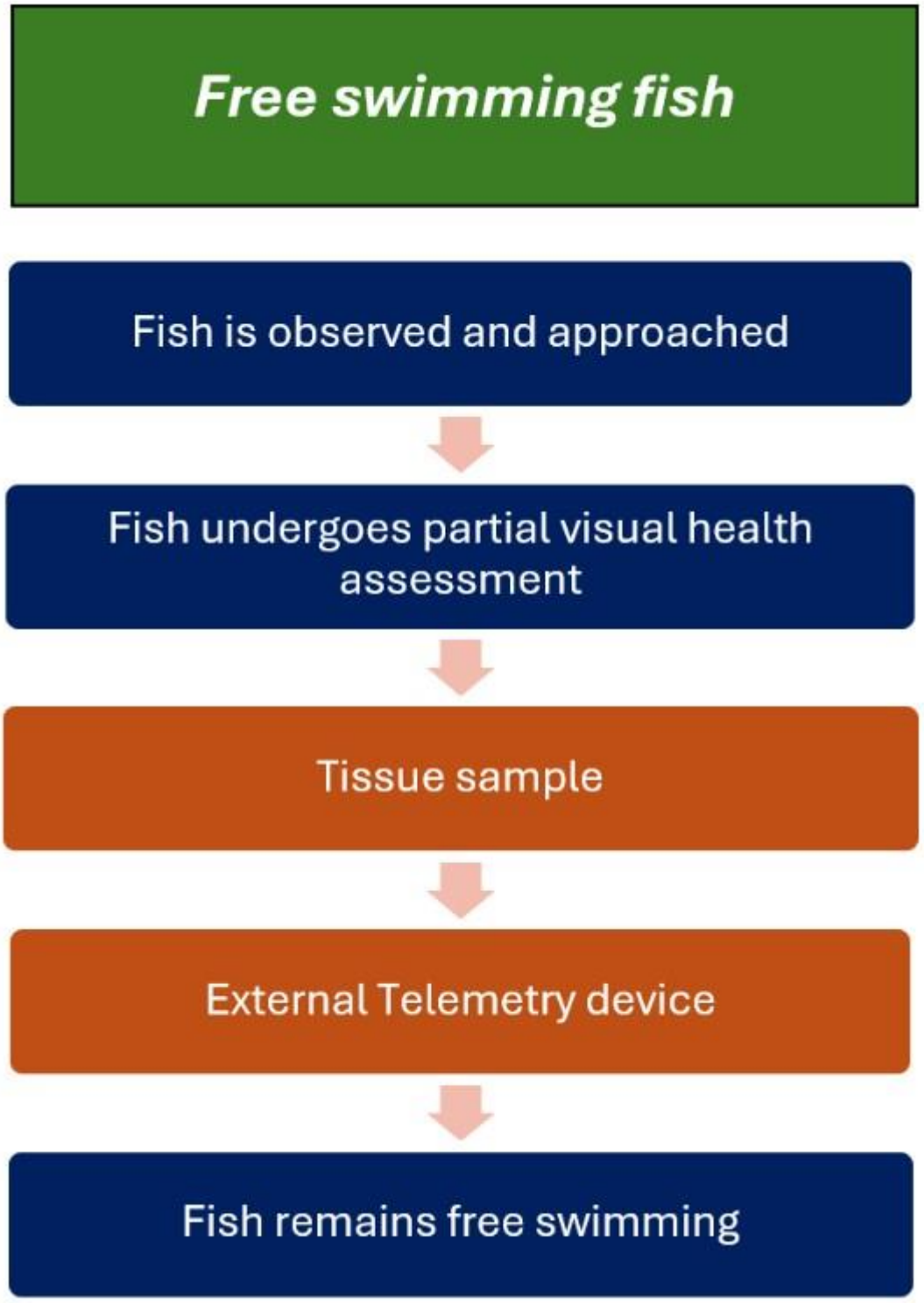
Explain why you are using these types of animals and your choice of life stages.

The work undertaken in the research programme that will take place under this licence will collect data on species of conservation concern as identified via international and national conservation listings (e.g., OSPAR, IUCN (International Union for Conservation of Nature), Convention for Migratory Species, Priority Marine Species lists). To effectively manage these species, gathering data on the specific species of conservation concern is crucial to inform effective management. Managing all life history stages is crucial to maximise recruitment and ensure population success. Due to many species' tendency to use different habitats and make different movements, it is essential that data is collected for all life history stages of a species to refine management decisions to as great an extent as possible to maximise its efficiency while having as little impact on the broader marine ecosystem and

stakeholder user groups as possible. Any unintentionally captured Endangered species (by the ASPA 1986 criteria) will be released (providing that their health and welfare allows this) without being subjected to any regulated procedures.

Typically, what will be done to an animal used in your project?


Compulsory Step
Optional Step
Fish is captured
Fish is landed
Fish is secured, but left in the water
Fish undergoes full health assessment
Blood sample
General anaesthesia
Local Anaesthesia
ID Tag
Local Anaesthesia
Fin Clip
Blood sample (if not already taken)
Local Anaesthesia
Tissue sample
Cloacal Swab
Ultrasound
Local Anaesthesia unless under general anaesthesia
External Telemetry device
Local Anaesthesia unless under general anaesthesia
Surgical tagging
Final Health Assessment (possible blood sample)
Fish is released OR Euthanised (no ID tag)



Typically, each individual fish in this programme of research will be removed from the water for short time periods (<5 minutes) to measure the animal (length, width, circumference), collect a tissue sample taken in the form of a fin clip or muscle sample (<0.5 cm3). Fish removed from the water for more than

30 seconds will be supplied with a flow of clean water over the gills to allow oxygen uptake. Fish subject to procedures will be placed on an appropriately shaped (species dependent) clean, soft surgical mat and are gently restrained using foam supports to

restrict movement and the risk to the animal and nearby people. See attached figures for flow diagram of fish experience for free-swimming and captured fish.

ID tagging: All fish in this project will be ID tagged so they can be uniquely identified. Depending on the species, this tag will either be an intramuscular Passive Integrated Transponder (PIT) tag or an external ID tag, such as a dart tag or a Peterson disc. PIT tags are tiny tags, about the size of a grain of rice, that are injected into the muscle of the fish. They are scanned using an external scanner unit (non-contact) to read the ID number. External tags have small, non-reactive nylon or metal anchors inserted into the musculature to hold the tag in place. The diameter of these anchors is not greater than 2 mm. Tag choice will be based on the species being researched (body shape and size) and the suitability of the tag technology (e.g., not all systems provide the opportunity for wide-spread PIT scanning to inform recaptures)

In addition to standard measurements (detailed above), some captured fish will undergo scientific research procedures. This will increase the amount of time they are out of the water, but during this time, they will be appropriately supported and supplied with a flow of clean water over their gills to facilitate oxygen uptake. Scientific procedures include:

Blood sampling: We will collect a blood sample from the fish. This will be drawn via a hypodermic needle and syringes from superficial blood vessels, for example, near the tail fin. This area is relatively easy to access and limits stress on the fish. A small species-appropriate-sized hypodermic needle will be used. A small amount of blood (species-dependent, total will not exceed 0.5 ml per kg bodyweight: Approximately 1% of total blood volume, based on 5% of total weight) will be taken, and the needle will be withdrawn. Gentle pressure will be applied to the site for ~1 minute to stem blood flow.

Tissue sampling: We will collect a small tissue sample for genetic and isotopic research. In most cases, this will involve removing a small tissue sample from the trailing edge of the fish's pectoral or dorsal fin. This method is commonly adopted by researchers worldwide and pain management is not used. For some fish, a small sample of muscle tissue will be collected using a biopsy punch (size dependent on the body size of the sampled fish); as chemical agents, such as anaesthetics, can affect the subsequent analysis, we cannot use a local anaesthetic before the sample is taken. However, for muscle samples, a local anaesthetic will be applied post-sampling to aid healing, and any resulting wound will be appropriately closed (e.g., with a suture).

Cloacal swab: A cloacal swab will be used to collect faecal samples for health and DNA analysis. A sterilised cotton swab will be inserted into the fish's rectum via the cloaca to obtain this.

External attachment of telemetry device: This research programme will also attach electronic tracking tags to some fish species. These tags will collect a high amount of data that allows us to recreate an individual’s movement and understand more about habitat use. The tag will either be attached externally by anchoring into the muscle or through a fin. Local anaesthetic will be applied at the point of tag attachment where possible. For remote tagging of free swimming species (e.g. basking sharks), administration of local anaesthetic will not be possible; this only applies to larger species that are less affected by external tagging.

Short-term deployments will use fin clamps

Intramuscular anchoring of external tag

Dorsal fin securement of external tag via a dorsal loop

Attachment of SPOT tag to the dorsal fin

Surgical tagging: Fish in a tracking study may be subject to surgical tagging. This reduces the chance of external irritation, tags snagging, being pulled out, and being removed by people encountering the fish. For this, the fish will be either under a general anaesthetic (administered via full immersion and absorption through the gills) or have a local anaesthetic (administered into the muscle at the site of incision) to mitigate stress and pain. In both cases, once anaesthetised, an incision will be made in the underside of the abdomen into the body cavity. Through this, we will insert an acoustic transmitter to transmit data for up to 10 years. This incision will be closed via absorbable sutures, which will fall out once the wound has healed.

General Anaesthetic: The fish will be placed in a dark dosing tank large enough to allow full immersion and space to move. The water will have been previously dosed with anaesthetic. Fish will be continually monitored until an appropriate level of anaesthesia has been achieved, determined by visual cues, including slowed gill/spiracle rate, cessation of movement, and compromised ability to maintain body balance.

Local anaesthetic: The area where the tag will be inserted is cleaned and disinfected to reduce the risk of infection. Anaesthetic is injected into the muscle around the identified surgical site using a thin needle (maximum 1mm external diameter). The amount of anaesthetic to be administered is measured beforehand based on fish size to prevent an overdose from harming the fish. The fish's vital signs and overall condition are monitored closely throughout the injection and subsequent surgical procedure to ensure its safety and well-being. Locally anaesthetised fish are released immediately unless complications require the fish to be monitored. Fish requiring monitoring after local anaesthetic and all those subject to general anaesthetic will be placed in a recovery tank and constantly assessed until deemed fit to release.

What are the expected impacts and/or adverse effects for the animals during your project?

There are several adverse effects that fish worked with during this research programme could experience. However, these adverse effects can be mitigated through the refinement of research methods, and these refinements are detailed below alongside the expected adverse effect:

Stress and Discomfort (<1hr): Handling procedures can induce stress and discomfort in fish. This stress response may vary depending on the species, size, and handling techniques used. However, using the refined procedures detailed in this application will limit stress and discomfort.

Physical Injury (2-3 weeks or longer): Improper handling or sampling techniques can lead to physical injuries such as tissue damage, punctures, or wounds. These injuries can impair the health and welfare of the fish over long time periods. The expertise of the researchers working under this licence, using the refined DOPs and SOPs, will significantly reduce this occurrence. There were no occurrences of this happening for work under a previous PPL (P05E95C50) the applicant held. For tagging procedures, the location of the tag is important for the comfort and health of the elasmobranch and the function of the tag. The location chosen for all internally inserted tags in this project is the peritoneal cavity, approximately midway between the isthmus and the cloaca. The risks of

internal organ damage due to tag insertion are low. An experienced tag surgeon, using a well-practised technique, will carry out the procedure. Special care will be taken when making the first incision through the skin to avoid cutting into any of the organs below, with special care taken to avoid damage to the liver, which, in all elasmobranchs, is an enlarged organ and can take up much of the peritoneal cavity space. Care will also be taken to avoid accidental suturing of any internal organ when closing the wound.

Behavioural Changes (<1 day): Fish may exhibit altered behaviour due to handling and tagging procedures. This could include changes in feeding patterns, swimming behaviour, or social interactions. We will use the most refined methods available to minimise the behavioural impact on the individual fish used in this research programme. Data collected by telemetry devices can be analysed for post-release variation. Any abnormalities shown in the data will be compared to the fish's experience from the point of capture to the point of release to help refine methods. Previous research has shown that behavioural changes do not last for more than 24-h (work under PPL P05E95C50).

Increased Predation Risk (1 yr+ or the lifetime of the tag): External tags or markings may make fish more conspicuous to predators, increasing their predation risk. The species we are working on are top-level predators in their respective ecosystems, so these effects are predicted to be negligible.

Infection and Disease (2-3 weeks): Good surgical technique will ensure the incidence of infection is low, and all blades, sutures, tags, and tagging equipment will be sterilised before use. It will be ensured that work will be carried out as aseptically as possible. The tagging equipment will be appropriate in relation to the size of the elasmobranch. An adequate number of individual stitches (normally 3 if implantation is intra-peritoneal, but with recent reductions in the tag size, it is effective to use just 2 sutures) will be made to ensure the internal tag wound heals well. In the wild, the wounds from tag implantation are expected to be healed after 1 month. In the case of externally attached tags, small lesions formed from scar tissue may occur on the skin at the point of entry of the tags or tag attachment wires. These are minor and often healed within a month. All equipment used for collecting samples is single-use and sterilised before use. The tools used for attaching and inserting tags are first autoclaved before being taken to the field. If they need to be used again, they are sterilised by physically cleaning them with alcohol and a brush. They are then soaked in sterilant for a minimum of 15 minutes and rinsed in sterile water.

Over/under anaesthesia (<1 day): Fish under anaesthesia will be constantly monitored (including heart rate in skate) to mitigate this adverse effect. Appropriate levels and types of anaesthesia will be used for the size and species used as per the projects SOPs developed with the NVS. The water for the anaesthetic and recovery tanks will be taken from the natural environment to ensure it has the same properties as the fish's natural environment (e.g., temperature, salinity, and hardness) and will be well-aerated. The anaesthetic water bath will be buffered to maintain pH. Any fish showing signs of abnormal swimming behaviour, distress, or abnormal respiratory movements in the recovery tank (after tagging) will not be released and will be humanely killed via a Schedule One method.

Healing from blood sampling/PIT tagging (<1 day): The small wound caused by the sterile sampling needle should heal <1 day.

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 (n=maximum 5000): This includes fish subject to ID and external telemetry tagging, fin clips, and blood samples.

Moderate (n=maximum 1000): This includes fish subject to internal acoustic tagging.

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 work in this project will contribute to the suitable management of the study species. Management decisions are based on evidence, which is underpinned by species-specific data.

Which non-animal alternatives did you consider for use in this project?

Where possible, pre-existing data will be used. This will be sourced via public databases and research collaborations.

Why were they not suitable?

The species in this project are primarily data deficient and lack the pre-existing data required to answer research questions.

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 maximum numbers mentioned in this document reflect the upper limit of the number of individuals we envisage working on over the 5-year period. As current project funding extends to at least 10 species, with additional funding already applied for, this project will work on multiple species over its 5yr lifetime. To prevent work on excess individuals, the numbers of each species worked on will be rigorously assessed to ensure that the data requirements are met but not exceeded.

Providing estimates of numbers is complicated due to work on this project covering multiple species over multiple systems. No more than 5000 fish will be used in the programme of research covered by this Project Licence. Factors including the catchability

of a species, seasonal variation in localised abundance, and the impact of climatic events (rainfall, ocean currents, etc.) can drastically change the number of fish present at a sampling site. This project aims to provide data and information on various fish species by sourcing funding. Funding has already been sourced to work on elasmobranchs (multiple species) and Herring (Clupeidae), and grants have been submitted to work on scombrids and gadoids. Each species will be assessed to determine the number of fish that will be subject to procedures, ensuring no needless harm nor detriment to welfare. Our goal is to minimise the number of instances by following criteria:

Most fish exhibit variations in their ecology due to different movements and interactions with various habitats throughout their life. Studying the individual in question is necessary for investigating these variations rather than inferring from other sizes and sex classes. However, due to the tendency of fish to form schools, we can assume that an individual's behaviour may be representative of others in the same age and sex class. This assumption allows for a sub-sample of a population’s life history class to be studied as a representative sample. To gain sufficient information on a population, based on the above, a planned tagging campaign can reduce the number of animals needed for overall population results by targeting specific numbers of certain sizes and sexes. The data from these individuals can produce models predicting the movement of populations and habitat distribution models. Reducing the need for future tagging work.

An additional measure to reduce the number of animals being tagged is to use the tags most suitable to the species (based on published literature and previous studies to maximise the chances of retrieving data while deploying the minimum number of tags. When deciding on the type of tag to use, we consider both the species being studied and the probability of recapturing the fish. If the likelihood of recapture is low, we can use satellite or acoustic-linked tags to obtain the maximum amount of data while minimising the number of fish that need to be tagged.

Where possible, when tags are available, and individual elasmobranchs are large enough, we will deploy more than one type of tag on an individual. This has two benefits: first, it increases the accuracy of space use models, and second, it reduces the overall number of individuals to be tagged.

Based on experience gained in previous research projects, capture equipment will be optimised to target specific fish sizes and reduce the bycatch of unwanted species. The location for capturing animals will be chosen carefully to ensure maximum opportunities for capturing the target species by sex and size. This approach will minimise the overall number of animals captured while ensuring a good range of sizes and sexes are appropriately tagged. This will reduce the number of individuals captured during this project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Using the refined methods developed during previous Licensed research projects, we will undertake as many procedures as ethically possible on each individual caught (without causing increased detriment to the individual's health or welfare). This approach will allow for better science and reduce the number of fish that must be captured during this project. Sufficient numbers of fish must be marked and recaptured to generate parameter estimates of sufficient precision to meet management needs. Given the size of wild populations and the complications in accessing aquatic species, it is highly unlikely that

any fish will be sampled and tagged unnecessarily. All data collected from all individuals will improve our understanding of how these species use their aquatic environments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Alongside collecting multiple samples from the same individuals, we will ensure that all collected samples are shared with other researchers working on the same species. We will contact these researchers prior to undertaking any sampling to ensure that the collection of additional tissues is warranted. Data on a few individuals can be used to produce models predicting the movement of populations and habitat distribution models. Reducing the need for future tagging work.

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 methods used during this research programme have been selected to minimise the amount of pain, suffering, distress, and long-lasting harm to all animals involved. They have been developed and refined through over 15 years of experience by the Project Licence holder and in collaboration with veterinary surgeons and experienced researchers working in the same field. By employing the latest technologies and best practices and using the most species-relevant equipment, we ensure that the welfare of the fish is a top priority, allowing us to gather essential data while minimising any negative impact on the animals involved.

Anaesthesia

Fish in this project will be tagged under either local or general anaesthesia. Local anaesthesia is the more refined method as it minimises systemic effects, provides targeted pain relief, allows faster recovery, and minimises chemical release into the environment. Local anaesthetic also offers pain management for species too big to immerse in an aesthetic bath to put under general anaesthesia.

Using local anaesthesia drastically reduces the time the fish spend outside their natural environment, reducing stress. However, while local anaesthetic is the more refined approach, there are situations where general anaesthesia is necessary and will still be used for sensitive species and life history classes. Some fish species and life history stages may be more sensitive to handling or environmental stressors, making them unsuitable candidates for local anaesthesia alone. In such cases, general anaesthesia may be necessary to minimise stress and ensure the welfare of the fish during surgery. In summary, while local anaesthesia offers several refinements over general anaesthesia in fish surgery by minimising systemic effects and providing targeted pain relief, there are situations where general anaesthesia may still be necessary to ensure the safety, success, and welfare of the fish during surgical procedures. The choice of anaesthesia method will

be based on careful consideration of the specific surgical requirements, fish species and body size, research objectives, and ethical considerations. These will be fully discussed with NVS and agreed upon before any work commences.

Tagging methods

ID Tagging: Using ID tags in fish research is crucial for identifying individual fish. ID tagging is the most refined method for individual fish identification because it is tailored to be minimally invasive and species-specific. By designing tags to be small and lightweight, researchers ensure that the tags do not significantly impact the fish's natural behaviour or comfort. The tags will be applied using minimally invasive techniques, ensuring that the tagging process causes minimal discomfort. Anchoring tags in the musculature or through the dorsal fins using minimally invasive techniques further minimises discomfort and stress. There are different types of tag available, and the tag choice will be made for each species and life history class with agreement from NVS prior to field work. For example, Petersen discs are not suitable for small species, so they will only be used on fish over a pre-determined total length.

External Telemetry Tagging: Telemetry tags are the only method of providing valuable data on fish movements and behaviour. External attachment is essential for some tag types due to the need to recover the tag to collect the data or the need for the tag to be 'visible' to satellites. Tag size will be chosen based on species size and morphology to ensure minimum impact while collecting the required data to meet the research objective. Tag attachment will be via either dorsal/wing (in skate and rays) muscle anchor, dorsal fin/wing (in skates and rays) through pins/loops, or fin/wing clamps. The attachment sites are selected to avoid sensitive areas, and the tags are designed to be hydrodynamic, reducing drag and interference with the fish's natural behaviour. Local anaesthetic will be applied at the tag attachment point (where possible – for example, this is not possible in free-swimming fish that are remotely tagged) to minimise stress and pain further. The procedures are relatively quick, reducing the handling time and associated stress. Note, in some instances, telemetry devices may require two anchor points in order to ensure suitable retention.

For external ID and telemetry tagging, some large fish (e.g., basking sharks or large tuna) may be tagged while the animal is free swimming. This is done to minimise the pain and suffering to the animal that would be caused if landing was attempted.

Surgical Telemetry Tagging: Surgical telemetry tagging allows for the collection of detailed

physiological data. This method involves implanting tags within the fish's coelomic cavity under local or general anaesthesia (size and species dependent). The surgical procedures are conducted by highly trained personnel using refined surgical techniques to minimise tissue damage and pain. The internal implantation of acoustic tags over external attachment for long-term tracking is preferable as external tags have the potential to move in the water as the elasmobranch swims, causing mild irritation to the skin and, in some cases, stimulating a healing response and the formation of scar tissue around the tag. Furthermore, external tags can either be removed or fall off, especially during long deployments. This limits the amount of data a tagged animal can provide and decreases the benefit of the project in a cost/benefit analysis. Internal tagging minuses the impact on the fish's natural swimming. For tags that will be internally implanted, the water weight will not exceed 1% of the fish weight or 15% of the fork body length. In each case, the smallest, least invasive tags available to collect sufficient data and have adequate battery life to yield robust results and answer the question will be used. Where possible, we will

monitor physiological indicators, such as heart rate (via non-invasive ultrasound) to better understand the impact of tagging. This will help us refine our procedural and handling protocols to ensure that we have as small an impact as possible on the animal's welfare.

Additional sample collection

Blood Samples: Collecting blood samples is the most refined method for obtaining vital information on fish diet, reproductive biology, and health because it is quick and minimally invasive. Blood will be collected from the caudal vasculature via a hypodermic needle (gauge is species dependent) and a pressurised syringe. The sampling techniques have been refined to be quick and minimally invasive.

Cloacal Swabbing: Cloacal swabbing is considered a refined method for collecting data in fish research due to its minimally invasive nature and ability to provide critical biological information without causing significant harm to the fish. The procedure is quick, requires minimal handling, and typically results in little to no discomfort for the fish. By reducing stress and avoiding more invasive methods, cloacal swabbing allows researchers to gather essential data while maintaining the well-being of the fish, making it an ideal choice for both routine monitoring and specialised studies.

Tissue Samples: Tissue sampling is important for genetic and isotopic analysis, disease monitoring, and environmental assessments. Tissue sampling is highly refined due to its optimisation for minimal invasiveness and species-specific considerations. Fin clips (<5mm2) will be collected from the trailing edge of the dorsal or pectoral fins or the trailing edge of the wing in skate and ray species. Muscle samples will be collected via a small punch biopsy. in species with tough skin (e.g., elasmobranchs), the skin will initially be cut using a scalpel. The methods for obtaining tissue samples have been optimised to use the smallest possible amounts of tissue, obtained through minimally invasive techniques using the most species-relevant equipment.

Ultrasound: Ultrasound is a non-invasive method used to study the internal structures of fish, such as reproductive organs and organ health. This technique involves using harmless sound waves that do not cause pain or distress. The most species-relevant ultrasound equipment is used, ensuring accuracy and comfort. The procedure is conducted with minimal handling, and the fish can often remain in water during the ultrasound, further reducing stress.

Why can’t you use animals that are less sentient?

The work carried out under this Licence will collect data on species of conservation concern, as identified via international and national conservation listings (e.g., OSPAR, IUCN, Priority Marine Species lists). To effectively manage these species, gathering data on the specific species of conservation concern is crucial to inform effective management. Managing all life history stages is essential to maximise recruitment and ensure population success. Due to many species’ tendency to use different habitats and make different movements, data must be collected for all life history stages of a species to refine management decisions to as great an extent as possible to maximise its efficiency while having as little impact on the broader marine ecosystem and stakeholder user groups as possible.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To refine procedures towards minimising the impact of procedural work on the animals involved in our research, we will implement the following strategies based on the current practices and available methods:

Continuous Monitoring: Collect data on the capture and on-board experience of each animal (e.g., time in trap/time on hook, time out of the water, air temperature, sunlight levels, samples collected etc.) and undertake complementary physiological monitoring (e.g., heart rate, blood chemistry) of the animals while onboard to detect and address any signs of distress promptly and what the causes of this stress are

Behavioural Observation: Analyse post-tagging behaviour from tagging data in relation the capture and on-board experience of each animal to investigate the main causes of unusual behaviour and how to improve recovery time.

Minimally Invasive Techniques: Continue to develop and refine research methods to ensure they comply with up-to-date literature and research. Ensure open communication with colleagues working in the same field to support this. Ensure the smallest possible samples are taken to gather necessary data.

Tag Design: Ensure we are using the smallest and most hydrodynamic tag designs to minimise the impact on fish behaviour and reduce physical discomfort from tagging.

Enhanced Health Screening: Based on our findings, refine the health assessment criteria to ensure only the fittest animals undergo tagging. Animals deemed unfit will be ID tagged and released immediately to prioritise their well-being.

By incorporating these refinements, we aim to minimise the welfare costs further and ensure that the animals involved in our research experience the least possible harm while still allowing us to collect valuable scientific data.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Through the PPL holder's 15 years of experience, they have worked on developing highly refined Standard Operating Procedures (SOPs) that cover the procedures in the PPL application. These SOPs have been developed in collaboration with the NVS, other NVS's at multiple institutions, external veterinary surgeons with expertise with teleost and elasmobranch species, and other researchers working on these species within the UK and globally. The SOPs that will be used in this project are dynamic and are continuously updated to ensure they are as refined as possible.

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, the PPL and project team will:

Conduct regular reviews of the scientific literature to identify new methods and technologies that can replace, reduce, or refine the use of animals in research. Summarise findings and assess their applicability to ongoing

Participate in subject-relevant conferences, workshops, and seminars focused on fish welfare and the 3Rs.

Collaborate with Experts and utilise existing relationships with other experts in animal welfare and the 3Rs. Collaboration with experienced researchers can provide insights into new methodologies and technologies that promote the 3Rs.

All project personnel will attend training, workshops and conferences related to animal ethics and the 3Rs.

Advancements in 3Rs will be implemented via:

Integrate new 3Rs techniques into standard operating procedures (SOPs) and research protocols. Ensure all team members are trained in these updated methods and understand their importance.

Provide ongoing training and education for all team members on the importance of the 3Rs and the latest advances in the field. Workshops, seminars, and online courses can help keep the team updated and skilled in new techniques.

Work closely with the NVS and other AWERB members to ensure that new methods are ethically sound and compliant with regulations. Regularly review and update ethical approval documents to reflect the incorporation of 3Rs advances.

Share successful 3Rs strategies and outcomes with the broader research community through publications, presentations, and collaborative projects. This contributes to the collective advancement of animal welfare in research.

By adopting these strategies, you can ensure that your research project remains at the forefront of ethical and humane animal research, continuously integrating the latest advances in the 3Rs to improve animal welfare and scientific outcomes.

96. Nanomedicine design and development

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

therapy, drug development, polymers, nanoparticles, drug targeting

Animal types	Life stages
Rabbits	Adult
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?

We aim to test medicine fabrication technologies aimed at improving medicine effectiveness and limiting medicine side effects. The overall aim is to significantly improve the efficiency of the medicines development pathway, provide more efficacious medicines for patients and in this way promote adherence to 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?

Drug development is incredibly inefficient. Only one in 5,000 molecules make it from concept to marketed drug and in the clinical trial stages of drug development there is a 90% failure rate as medicines fail to show their much promised efficacy.

Medicines must be discovered by first discovering the active ingredient and then once an active ingredient is identified it must be developed into a medicine (e.g. a tablet, injection, eye drop or suppository). Sometimes the nature of the active ingredient makes it hard to make it into a medicine because it is fragile and easily destroyed when taken or because it cannot get inside the brain to treat brain diseases or it cannot get to the retina to treat diseases of the eye. Sometimes cancer drug active ingredients are hard to develop into medicines as they need to get in large quantity to the cancer itself and not to anywhere else. This is because when cancer drug active ingredients end up in healthy tissue, this causes side effects.

Putting medicines into tiny particles made of other materials (e.g. fats, carbohydrates and proteins) helps these active ingredients get to where they need to go in the body.

We wish to study how these tiny particles work and how they shuttle drugs around the body so that ultimately we can design particle-based medicines that are more efficacious and have less side effects than existing therapies.

We have already used our knowledge of these tiny particles to create a pain medicine candidate and licensed this to a United States (US) company, for further development. This company will test this medicine in humans and see if it can replace the opioid pain killers (e.g. morphine and fentanyl) used currently. 80,000 people die every year in the US from using opioids and 15,000 die every year in the US from using prescription opioids to control their painful conditions and so a safer replacement will be welcome and extremely timely. In addition 46% of the overdose deaths in the UK are due to the use of opioids and 40 people die every week from opioid poisoning in the UK.

Furthermore we have used these tiny particles to create various eye care medicines that are due to enter clinical testing shortly. One has been trialled in healthy volunteers in 2024.

What outputs do you think you will see at the end of this project?

At the end of our project, we expect to have learnt more about the way these tiny particles work in the body as this will help us design new and efficacious particulate based medicines with fewer side effects. We have already developed particle-based medicine candidates that are scheduled to undergo clinical testing by ourselves and by others.

Who or what will benefit from these outputs, and how?

We have already prepared medicine candidates based on these nanoparticles and these medicine candidates are due to enter clinical testing shortly.

In the short term (2 - 5 years), our work will benefit other scientists as they will learn from our published findings. Our work will also benefit the scientists working on our programmes as well as ourselves as we will all learn something new about the way these tiny particles work as medicines.

In the medium term (5 - 10 years), our work will benefit the economy if new medicines are developed or are in the process of being developed. These discoveries will lead to money coming to the university when these discoveries are out-licensed to third parties.

Ultimately in the long term (10 - 15 years), if new medicines are developed and approved for sale, our work will benefit patients who suffer from chronic pain or patients who are at risk of blindness. Finally our work may even benefit patients with breast cancer, prostate cancer, liver cancer or lung cancer.

How will you look to maximise the outputs of this work?

We will work with other scientists and engineers to make sure that we fully understand what these tiny particles are capable of as medicines. For example, we already collaborate with physicists as well as with eye doctors. These collaborators help us to understand how the formulations that we are developing may be used (e.g. clinicians) and help us understand the mechanisms of action of various potential medicines (e.g. physicists working with us on gold nanoparticles).

We will make sure that any findings that may be used to make new and improved medicines are taken forward either by establishing a new company or by licensing our findings to an established company so that the medicines may be developed.

We will publish all of our work, even when the results of the experiments show that the tiny particles are unable to shuttle active ingredients to particular areas. This will help scientists understand these particles better and inform others on which avenues to pursue within the discipline.

Species and numbers of animals expected to be used

- Mice: 3000
- Rats: 2000
- 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.

We need to study where the tiny particles go in the body and so we are using adult mice, rats and rabbits as these are the lowest vertebral life forms that are able to provide us with meaningful results.

We need to study how the medicines made from these tiny particles work and so we are using adult mice, adult rats and adult rabbits, as these are the lowest vertebral life forms that are able to provide us with meaningful results. Additionally our work targets adult diseases.

Typically, what will be done to an animal used in your project?

The animals will be bought from a company licensed by the Home Office and then transported to our animal house where they will live for 7 days in order for the animals to get acclimatised to the new environment. Environmental enrichment will be in the cage so that the animals are not bored.

On Day one of the experimental phase of the project, the animal will be dosed some of our tiny particles and the dose will be such that it is unlikely to cause harm as the particles would have been tested in cells first. The animals may be dosed more than once and the

dose volumes will be within the acceptable limits. Animals may be dosed with solids or liquids and via different routes. The animals will be killed after a suitable period of time and their organs and blood taken for analysis. Sometimes, post dosing, blood will be taken repeatedly from one animal over a one or two day period but this will be no more than 10% of the total blood volume over a 24 hour period and no more than 15% of the total blood volume over a 48 hour period. The animal will be humanely killed at the end of this study.

Some animals (mice and rats only) will be disease models (e.g. a pain model to test if the analgesics are working) and so they may be subjected to moderate pain in order to see if the tiny particles reduce this pain. A typical pain experiment, known as the Tail Flick Test, may involve the animal having its tail dipped into hot water of 50 degrees centigrade. 50 degrees centigrade is just hot enough for a human to immediately withdraw their hand but not hot enough to cause scalding. The animal will be able to immediately withdraw its tail from the water if it feels painful and will keep its tail in the hot water longer if the tiny particles are giving the animal pain relief. Another typical pain model is a nerve damaged model. In all cases we will measure the pain relief felt by the animal. The animal will be humanely killed at the end of this study.

Only healthy rabbits will be used for our studies.

What are the expected impacts and/or adverse effects for the animals during your project?

We intend to obtain results which will tell us that the tiny particles are working in the animals either to bring about relief from a disease state or that they go to areas in the body where we want the particles to go, such as the site of the disease.

Some animals may suffer weight loss of more than 15%, moderate pain, show signs of discomfort by remaining hunched over or make unusual sounds. We do not expect more than 5% of our animals to show these effects.

Some animals may suffer from the anaesthetics that we give them and have an unexpected effect that shuts down their organs. We do not expect more than 2% of our animals to show these effects.

Common and milder effects like bruising after we have withdrawn blood may affect about 10% 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)?

We expect 90% of mice to have mild effects and 10% of animals to have moderate effects.

We expect 90% of rats to have mild effects and 10% of animals to have moderate effects.

We expect 95% of rabbits to have mild effects and 5% of animals to have moderate effects.

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 tiny particles that we are testing are carried by the blood to the various organs and so we need to test them in the presence of flowing blood. This can only be done in live animals.

Which non-animal alternatives did you consider for use in this project?

We considered the use of computer models and organs grown in laboratories.

Why were they not suitable?

The computer models do not accurately mimic blood flowing in a mammal and the models do not thus mimic the interaction between blood flow and the target cells of interest. Organs grown in the laboratory do not yet have a working blood supply.

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 stated are the upper limits for our animal numbers.

We will always first conduct a pilot experiment to measure the size of the response and by how much it varies and this will be used to decide how many animals are needed in each group. The animal numbers in each experiment, come from experiments that have been conducted in our laboratory previously. Group sizes have been determined using statistical methods and analysis.

Mice - For group sizes of at least 5 animals and with a test and one control group and sampling at 8 time points, we estimate that each experiment will need about 100 animals (including the pilot studies) and we will conduct about 6 experiments per year and hence will need 3000 over 5 years.

Rats - For group sizes of at least 5 animals and with a test and one control group and terminal organ sampling at 6 time points, we estimate that each experiment will need about 80 animals (including pilot studies) and we will conduct about 5 experiments per year and so we will need 2000 over 5 years.

Rabbits - For group sizes of at least 5 animals and with a test and one control group and terminal organ sampling at 4 time points, We estimate that each experiment will need 50 rabbits and we will conduct about 2 experiments per year and so we will need 500 rabbits over 5 years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We need each experiment to yield meaningful data that we are able to use. This will limit the overall number of animals used, as experiments then do not need to be duplicated.

We used published guidelines to design our experiments (e.g. the PREPARE guidelines and the NC3Rs' Experimental Design Assistant).

We have used power calculations to make sure that our experiments contain enough animals so that we are able to detect any differences in our 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 will carry out pilot experiments to see how big our signal is and by how much it varies. We will then use this information to calculate the best group size for our experiments that will give us reliable 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.

We will use healthy mice, rats and rabbits to study where the tiny particles go in the animal. These models are best as there will be no presence of disease in these models and we will make sure that the dose that we select for our main studies have been shown not to cause harm in a pilot study of a few animals.

When we want to measure whether the particles work in the animal as potential medicines, we may use disease models, but these disease models will be such that the disease/condition does not cause severe harm.

We plan to use pain models but have chosen pain models that do not cause suffering. All of the pain models that we will use cause the least pain and suffering for the animals, but allow us to know if the medicines that we are developing for painful conditions actually work.

Why can’t you use animals that are less sentient?

The tiny particles need to be tested in mammals with a blood flow that is comparable to that found in human beings.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will minimise animal suffering by making sure that the tiny particles that we give the animals have first been tested on cells and will give them at a dose that theoretically will

not kill the cells in the first instance. We will then take the dose up slowly observing for any harm along the way and stopping the experiment if we observe harm that is above our moderate limit.

We will observe all animals for a period after dosing to make sure that we can pick up any unexpected bad effects. In any event we will check our animals daily if we are doing longer experiments.

With any surgery or other use of anaesthetics we will monitor the animals after the procedures to make sure that we pick up any unexpected bad effects.

Apart from when specifically testing in a pain model. we will give the animals analgesics if we observe them to be in pain (e.g. after surgery) and will give the animals analgesics beforehand if we anticipate any procedures may cause pain (e.g. before surgery).

All surgery will be done under general anaesthesia.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the ARRIVE and PREPARE guidelines.

We will use the LASA Administration of Substances guidelines.

We will use the LASA Guiding Principles for Preparation 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 read the literature and monitor updates to the NC3R, PREPARE and ARRIVE guidelines. These are laboratory practice guides that tell one how to conduct animal experiments in a way that minimises animal harms.

97. Understanding the mechanisms of retinal vascular remodelling in diabetes

Project duration

4 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

Diabetes, Ageing, Blood vessels, retina, therapy

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 project's objective is to improve our comprehension of the molecular processes accountable for impairment and loss of vision during diabetes. Our focus will be on examining the function of a protein we have recently identified that is associated with these modifications and on the identification of new ones.

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 although there are some treatments available for patients with diabetic eye disease, a substantial number of patients fail to respond to current

therapies (~50%). Also, these therapies are only effective at the late stage of the disease when sight impairment has already occurred. The work planned here will shed new light on the early events of diabetic eye disease and directly support work in our laboratory aimed at developing novel early therapeutic approaches.

What outputs do you think you will see at the end of this project?

The main output of this research will be novel insights into the molecular mechanisms driving retinal vascular pathology in diabetic eye disease. Likely, this new information could also be applicable to other vascular complications of diabetes, including diabetic kidney disease.

We expect that these new findings will be shared primarily through peer-reviewed research publications, seminars, press-releases and patient/public engagment events.

Who or what will benefit from these outputs, and how?

We will share our research discoveries and outputs to the scientific community as soon as we obtain them, hence some will be within the course of the project and some straight after.

We will welcome and actively seek opportunities for patients and public engagement through diabetes and vision charities, our university and external bodies, during and after the course of the project.

If the project is successful, new therapies based on our discoveries might reach clinical trials between 5-10 years after the end of the project.

How will you look to maximise the outputs of this work?

Publication in open-access journals, presentation at international scientific meetings, making resources available to other researchers (e.g., data, animals, tissues).

Species and numbers of animals expected to be used

- Mice: 16000

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 have proven invaluable in advancing our understanding of the mechanisms underlying pathological changes associated with diabetes. Here we will primarily use two models, namely the Ins2AKITA mouse and the streptozotocin (STZ) mouse. The use of two models significantly strengthens the value of any scientific outputs by obviating potential criticisms that observations may be model or strain-specific. In both models changes to the retina become manifest in adulthood and resemble closely the changes observed in patients with early DR.

Typically, what will be done to an animal used in your project?

We will observe the mice until they have had diabetes for a set period, after which we will either humanely euthanise them to study their tissues, or we will assess the structure and function of their retinas while they are under general anaesthesia using methods similar to those used on human patients. In some experiments, we will administer treatments directly into the eyes of anesthetised mice. These treatments may include antibodies designed to block the function of a protein. Depending on the specific experiment, a mouse might receive just one treatment or multiple treatments at fourweek intervals over three to four months to assess the longer-term effects. We do not expect to perform any surgical procedures.

What are the expected impacts and/or adverse effects for the animals during your project?

We have worked extensively with diabetic mouse models, and have not observed problems with animals experiencing pain, tumours or abnormal behaviour. In these mice, insufficient insulin production results in less weight gain compared to healthy mice because their bodies cannot absorb glucose properly. This leads to higher blood sugar levels, making the mice drink more water and urinate more frequently, necessitating daily changes of their bedding. These side effects begin when the mice become diabetic and continue for the rest of their lives.

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 levels in the diabetic mice are expected to be moderate, and we would anticipate this to apply to all diabetic animals. Healthy animals used as controls and transgenic animals without a harmful phenotype will be mild severity. The expected proportion of mild and moderate severity is 50:50.

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 complexity of diabetes cannot be fully replicated in cell culture models or even explants. Only in live animals, with a fully functional vascular system, can we observe the intricate changes in the retinal vasculature that mirror those seen in human diabetic patients. These changes involve various retinal cell types that interact and communicate in

ways that are so intricate and interdependent that no current in vitro system can accurately replicate them.

Which non-animal alternatives did you consider for use in this project?

Human Retinal Microvascular Endothelial Cells (HRMECs): These cells can be cultured in vitro and used to study various aspects of retinal vasculature, including angiogenesis, permeability, and response to diabetic conditions.

Pericyte-Endothelial Cell Co-Culture Systems: This model mimics the cellular interactions between endothelial cells and pericytes, which are critical in maintaining the blood-retina barrier and are affected in diabetic retinopathy.

3D Retinal Organoids: These are more complex, three-dimensional structures derived from stem cells that can recapitulate the architecture and function of the retina.

Why were they not suitable?

While we can and do use in our lab cells in a plate to study some aspects of blood vessel structure and behaviour, there is currently no methodology to create in the lab a functional and perfused vascular network, we therefore have to rely on mice for these 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 proposed animal numbers are based on the usage from our previous project licence for the same protocols. We have also used a range of experimental design tools and resources (such as NC3R's Experimental Design Assistant) to determine the correct experimental group sizes to minimise the number of animals (e.g. careful consideration of control groups). Data from pilot studies will be analysed and methods refined where necessary before continuing the study in larger cohorts of animals. Calculations typically show that we need group sizes of 8-10 to achieve the quality of results we need. We have 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 employed several aids including SyRF and PREPARE (Smith AJ, Clutton RE, Lilley E, Hansen KEA, Brattelid T. PREPARE: guidelines for planning animal research and testing. Lab Anim. 2018 52:135-141) to reduce unnecessary animal usage by ensuring that the appropriate model system is chosen and that an appropriate and robust sample size is employed.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For wild type mice, we will breed or purchase only as required. Where we breed genetically modified mice, surplus wild-type mice not needed as littermate controls will be used for wild-type studies. When using new reagents, we will optimise the dosage in a small number of animals through pilot studies. Whenever possible, we will bank tissue that is not subject of this study and make it available to other research 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.

The mouse models chosen for the investigation of vascular pathology in diabetic eye disease are wellestablished and have been extensively characterised by research groups world-wide, including ours. Using such widely used models allows us to draw on a vast array of additional published data and reduces the need for additional experimental validation. Diabetic mice typically exhibit minimal changes in welfare, with the major management issue being provision of wet food in the cage and adequate provision of water to meet the increased thirst. This in turn necessitates more frequent changing of cage bedding (daily) and closer inspection (daily), something we have managed without problems for many years. Our animal unit and researchers are trained in recognising signs of discomfort in diabetic mice.

Why can’t you use animals that are less sentient?

Mice are the simplest vertebrates that possess an adult retinal structure closely resembling that of humans, including many of the same cell types, structural characteristics, and cellular functions. Less sentient animals such as Zebra fish, yeast, C. Elegans etc, simply do not have the vascular structures that permit an accurate modelling of human diabetic disease. Similarly embryonic animals or animals under terminal anaesthesia cannot be used because of the duration of elevated blood glucose required for pathological changes to occur. The mouse models selected for this project precisely replicate the disease that leads to blindness in humans. This alignment ensures that the research findings will effectively meet the project's objectives, advancing our understanding of diabetic eye disease and contributing to the development of new therapies to treat blindness.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Diabetic mice typically exhibit minimal changes in welfare, with no reports of pain, and the major management issue being adequate provision of water to meet the increased thirst and urination typically seen in these animals. The increased urination in turn necessitates more frequent changing of bedding in cages (daily), the use of more absorbent bedding

and closer inspection (daily) by trained staff, something we have managed without problems for many years.

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 all mice will be monitored regularly to ascertain health status and condition. In all cases animals will be weighed frequently and overall body condition will be used to determine health and establishing endpoints (e.g. see: ILAR Journal V.41 (2) 2000 Humane Endpoints for Animals Used in Biomedical Research and Testing). At any time the NVS and NACWO will be consulted if there are any questions. Researchers and animal staff will be instructed on specific symptoms, and given instructions on humane end-points. We will follow 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 an The ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines are intended to improve the reporting of research using animals – maximising information published and minimising unnecessary studies (https://www.nc3rs.org.uk/arrive-animal-research-reporting-vivo-experiments).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our institute has a NC3Rs advocate who regularly disseminates new information about experiment design, methods and replacement technologies to all investigators involved in animal research. The scientific literature is continuously scrutinised and discussed by academics at the institute through informal lab meetings, journal clubs and seminars. We will strive to identify and implement new techniques that could promote the 3Rs. Implementation of any new published and validated advances will be undertaken, where necessary through modifications to this Project Licence.

98. The use of mouse models to develop better treatments for advanced prostate 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
    - 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

Cancer, Prostate, Therapy, Immune response, Microbiome

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 project aims to use mouse models to better understand how prostate cancer is impacted by a patient’s immune system, nutrition, and microbiome (bacteria within the body that, along with other functions, aid food digestion) and to use this knowledge to develop better and kinder treatments for the benefit of prostate 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?

Background: Prostate cancer is the most common cancer in men living in Western countries. Although treatments for localised prostate cancer are effective, relapse is common and advanced disease, when the cancer spreads outside the prostate gland, remains lethal. The advanced stage of prostate cancer spreads throughout the body, often and very painfully to the bone, but also to the lymph nodes, the liver and other organs. Treatment options for the advanced stage of cancer are limited and mainly palliative. To find a cure for this detrimental disease, a better understanding of how prostate cancer develops and resists treatment, is an urgent unmet medical need.

For decades, cancer, including prostate cancer, has been looked at as a contained disease system, researched in isolated cultured cancer cells. It was long ignored that the tumour relies on the patient's body for blood support, food, and energy. Furthermore, research by our lab and others has shown that the gut microbiome (e.g., the bacteria in our gut helping our body to digest food) and the immune system can be 'hijacked' by the tumour in a way that helps the tumour to grow, survive and resist treatment. This work has shown that the microbiome can support tumour growth either directly, by contributing to hormone production and providing essential nutrients for cancer growth, or indirectly, by influencing the immune system. Both bodily systems have been shown to determine response to cancer treatments and predict disease progression and survival.

Working Hypothesis: Recent research by our team, and many others, has shown that prostate cancer is not a disease in isolation and that its development requires interaction with the rest of the body, in particular the microbiome in the gut and the immune system. We therefore hypothesise that targeting these interactions is a promising strategy to improve anti-cancer treatments and decrease side effects. Understanding the underlying mechanisms will lead to a better understanding of the disease and ultimately to improvement of patient care.

Experimental Strategy: Our group has developed a strategy to grow small prostate cancer pieces from patient biopsies in mice first, to form larger tumours, called patient-derived xenografts (PDX) which can be further processed to grow as cell lines in petri dishes and in mice. So far, we have developed over 40 new models covering the most relevant types of treatment-resistant prostate cancer. These have been used in various studies and have already greatly contributed to the field. We will now optimise these models by studying the impact of such as metastasis (spread to other organs), the influence of immune cells, nutrition, and the gut microbiome on treatment response. These refined models will help us to understand how resistance can be either reversed or, better yet, prevented. We will use these models to develop better treatments and to identify ways to modify nutrition, the patient's microbiome, and the patient's immune response to support the effectiveness of those treatments. The knowledge obtained in this research will help with the design of novel clinical trials and will ultimately benefit our patients.

What outputs do you think you will see at the end of this project?

We will have developed better models to study advanced prostate cancer: We will use our expertise and access to donated patient samples to improve our tumour models further. This will include modelling of the advanced prostate cancer's ability to spread to distant organs (to 'metastasise') and its dependence on the immune system and microbiome.

We will have characterised these models and gained a better understanding of the biology of advanced prostate cancer: We will characterise these models to understand better how these cancers become resistant to treatment and to develop ways to reverse resistance.

We will develop better treatments for advanced prostate cancer: We will then use the models in vitro (in Petri dishes) and in vivo (in mice) to test new therapeutics, which can be drugs, supplements, or optimised diets.

We will have better markers to find the right treatment for each patient: We're working on finding the best treatments for each patient by using mice that either lack or have extra copies of specific genes. By studying these mice, we can confirm how important these genes are for prostate cancer growth and patient survival.

The combined knowledge will help us to develop new treatment strategies in a more holistic approach to treating the disease.

During the project, we'll collaborate closely with other research teams, the pharmaceutical industry, and hospital clinicians. This collaboration aims to enhance patient care as our ultimate goal.

Who or what will benefit from these outputs, and how?

Within a short time of the commencement of the licence and continuously throughout its term, it will benefit the field by providing clinically relevant mouse models of prostate cancer to support research that will ultimately have a high impact on patient treatment.

In the mid-term, our research will help better understand how the cancer hijacks the patient’s biological processes to promote tumour growth and progression. We will use this to optimise the patient’s diet and gut microbiome for better health and higher response rates to treatments.

At the end of this project, we will have gained and published knowledge on how the surrounding stroma (cells around the cancer), infiltrating immune cells and the microbiome support the growth and progression of the tumour and which treatments will be most successful in blocking this advantage.

The information we gather will guide our decisions in the clinic, shaping our approach not just in the next five years but also in the future. It will pave the way for planning clinical trials aimed at developing improved and more compassionate treatments for our patients.

How will you look to maximise the outputs of this work?

To ensure the maximum benefits for the outcome of this project, we believe the results, and mice developed under this licence, need to be shared and discussed with experts and the public. Our group, therefore, reports regularly at key national and international scientific meetings as well as in highimpact journals.

Where appropriate, we will also discuss our research outcomes with pharmaceutical partners and academic colleagues to ensure our research findings are developed into clinically relevant treatments for patients with prostate cancer.

Our research group also regularly hosts meetings with patients, their families, and patient advocates to update them on our research projects. The results from this project will be reported to them through these open meetings with the community which we serve.

By making our models accessible through our collaborations with other researchers in academia and industry, we will speed up progress and reach our goal of curing prostate and other cancers faster. **Species and numbers of animals expected to be used**

- Mice: 14100 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 genes of mice share approximately 98% similarity with those of humans. It is possible to induce human-like prostate cancers in mice by deleting or elevating the expression of certain genes in the prostate. Alternatively, certain mouse strains, due to genetically impaired immune systems, allow for them to host and grow human tissue (e.g., tumours from patient biopsies) or immune cells. Prostate cancer, like most cancers, is a disease of older age and needs time to develop. This is why we need, in some cases, to implant tumours into juvenile mice and/or allow the mice to reach an old age. In collaboration with clinicians at the hospital, we have already used this feature to develop more

accurate models of tumours in patients that can be treated with different therapeutic regimes. We would now like to use this knowledge to expand our portfolio by remodelling the impact of the microbiome and the immune system, as both have been recently identified to support prostate cancer growth and are therefore relevant treatment targets.

Typically, what will be done to an animal used in your project?

Our team has developed a method to generate patient-derived tumour xenografts (PDX). These are implanted and grown under the skin or inside the prostate of mice and harvested for further analysis in the lab before they severely affect animal wellbeing. To mimic metastasis, we will in some cases inject the tumour cells into the blood stream. If we study the impact of specific genes or the impact of immune cells, we will use mice that have been genetically modified to get prostate cancer faster. If a humane immune system is required, we will co-transplant human immune cells or immune cell precursors. For this, radiation might be used to minimise rejection.

To mimic the effect of androgen deprivation treatment (drugs that lower testosterone in the blood of the patient), a common first-line therapy in patients, some mice will be castrated to reduce the male hormone testosterone in their bloodstream.

To investigate the interplay between the gut microbiome and cancer, some mice will be treated with high-dose antibiotics to help cleanse their gut microbiome and replaced with relevant gut bacteria to study their function.

We will monitor cancer growth using non-invasive methods, such as calliper measurement, palpations and imaging techniques, such as MRI or ultrasound. This will also allow us to observe potential adverse effects earlier and end the study before the mice suffer unnecessarily. When assessing treatment in mice for the first time, we will perform a small pilot study within a range of drug doses that should not cause harm, to explore its possible side effects. We will carefully choose drug doses that are effective while minimising side effects. Mice will be kept using the highest standards of animal husbandry. We will plan procedures to avoid pain and distress and observe animals daily using trained staff. We will use painkillers (anaesthetic and analgesic regimes) and appropriate humane methods of killing. Treatments are applied by mouth or by injection. Before and throughout the treatment, cancer growth and animal well-being are monitored regularly. As prostate

cancers grow very slowly, treatment will be continued over several weeks up to several months.

What are the expected impacts and/or adverse effects for the animals during your project?

Many of our experiments will involve mice that will develop cancer, either under the skin, in the prostate or as metastasis in their internal organs or the bones. A local tumour (a cancer mass) can spread (metastasise) and thereby lead to obstruction of the bladder or may hinder the mouse's ability to move or breathe or impact the function of other vital organs.

Prostate tumours grow relatively slowly and take up to 9 months to form. We will therefore take extra care of our mice, and our trained personnel will monitor them closely under the supervision of a veterinarian. Mice will be checked regularly for signs of tumour growth and will be weighed once per week. For most of the time, our mice will not be overly affected by their tumours, however, when tumours breaches a certain size limit (usually 12-14 mm), animal welfare can be affected. Tumours will therefore be grown up to a specified size limit to ensure they do not interfere with normal activities and behaviour. Most mice will be humanely killed at this time point to study their tumours and other tissue in the lab. Prior to reaching a defined humane endpoint, some mice will undergo tumour removal and will be closely monitored to study metastasis.

Some treatments will cause side effects, such as weight loss. Radiation, in general, can cause skin damage or loss of muscle weakness. In particular, targeted radiation can cause local burns or radiation damage in the area treated. Total body irradiation can also impact the gut flora or the immune system, manifesting in diarrhoea, weight loss and accelerated ageing.

Immune cell transplants can cause unwanted immune reactions and gut transplants can cause diarrhoea or dehydration. We will closely monitor the mice and humanely kill them if we can’t treat them or if we are concerned about 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)?

The severity of the experiments will be moderate (74%) or mild (25.5%). In rare cases, (<0.5%) mice might die of natural or reasons unrelated to the experiments

The expected number of mice found dead is higher since we are using highly immunocompromised mice and keeping them longer than usual since patient-derived tumours grow very slowly and take long (months, in some cases well over a year) to grow.

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?

We aim to gain a holistic understanding of the impact of the whole body, in particular the gut microbiome and the immune system, on the formation and treatment response of prostate cancer. Our team is constantly developing, and optimising methods designed to study individual aspects of this complex interaction in vitro (in petri dishes). However, there are limitations in their ability to predict clinical responses and we need to use a biological system that is closer to the human patient.

Mice have been proven in numerous studies to be suitable for investigating human diseases and effective predictors of clinical response to treatment. We will use immune-deficient mice to transplant human tumour pieces and immune cells as the body's immune system will otherwise reject the human transplant and genetically modified mice to study the disease in a more natural environment.

Which non-animal alternatives did you consider for use in this project?

We made great progress optimising in vitro assays (tests in Petri dishes) using isolated cells from our models and culturing them in the lab. These can be used to study the biology and response to various treatments. We also developed methods to grow them in more tissue-like 3D cultures and combine them with cultures of immune cells or supernatant (liquid) produced by other cultures.

Why were they not suitable?

Despite these efforts, these models have many major limitations such as their limited lifespan, lack of blood supply and isolated nature and key questions are best studied in an organism. Although we have made great progress in developing culture techniques, the in vitro life span of these cultures is limited, and we still need mouse models to propagate (keep) the lines and ultimately test potentially promising treatment strategies in an in vivo model (mice) that reflects the tumour structure and dynamics in the patient better. On this licence, we will continue to make these models better e. g. by extending their life span or by developing better models using co-cultures with immune-cells or components of the microbiome. In addition, we will integrate more recent technical advances such as modelling and in silico studies to reduce animal use for science e. g. to inform the study design or to select optimised treatment regimens.

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 based on accurate statistical planning with the help of our statistician. To accurately calculate our numbers, we used data obtained on previous project licences.

Geneticly modified lines of low use will only be bred when required and active mating will not be constantly maintained. Where possible, the embryos or sperm of a line will be frozen to avoid unnecessary breeding.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our work is supervised by a trained statistician who is part of our research team and will help us to analyse the experiments, and to ensure that the optimal number of animals per experimental arm is used to obtain a statistically accurate and relevant result. If the impact of a compound on overall wellbeing or the efficacy in mice is not known, we will perform small pilot experiments with reduced group sizes before a larger-scale experiment. We aim to use state-of-the-art experimental planning tools such as the NC3R's Experimental Design Assistant to ensure that we achieve statistically robust and relevant results with the lowest number of animals possible.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We carefully design and conduct experiments to extract the most comprehensive and valuable data from each one. By doing this, we aim to reduce the need for repeating experiments, which helps us save time and resources. We utilise the best experimental techniques to minimize the number of mice needed and ensure reliable results. By controlling factors that might introduce variability, we enhance the consistency and accuracy of our findings. We use advanced, non-invasive imaging methods to track tumour development over time. This approach allows us to avoid sacrificing mice at various stages of the experiment, further reducing the number of animals used.

In summary, we focus on extracting maximum information from each experiment while minimising animal use and experimental variability, thereby improving the efficiency and ethical standards 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.

Wherever possible, we will utilise *in vitro* (in petri dishes) assays (experimental tests) to address scientific questions and optimise treatment regimes. However, as these are limited in complexity, they often fail to accurately predict clinical responses as they lack important features such as the microbiome, immune response, or blood vessels. We therefore need to use mice at the late stages of our research.

For our studies on human cancers, we will implant and grow small pieces from patient-derived cancers into mice with impaired immune systems (immune-compromised) as they do not reject the foreign tissue. To better understand and develop treatments that prevent the formation of metastasis (tumour spreading throughout the body) we will inject the human tumour cells (coming from mice or culture) into the bloodstream.

We know that tumour cells 'high-jack' the microbiome and the immune system to support their growth and resistance to treatment. To study the impact of immune cells and the

microbiome, we need to implant immune cells and/or change the microbiome directly or indirectly by changing the diet of our mice. Immune cell transplantation can cause side effects such as graft vs host disease, when the immune cells recognise the host as foreign leading to rejection or potentially blood poisoning. Microbiome modulation by food or probiotics in immune deficient mice can lead to diarrhoea and dehydration. To avoid side effects and minimise harm caused by human samples, we will health screen donor material and run pilot tests with smaller cohorts of mice.

To avoid side effects like rejection of human tissue, we will wherever possible use mice with an intact immune system, that have been genetically modified to form cancers faster.

Mice have been proven in numerous studies to be suitable to investigate human diseases and to be effective predictors of clinical response to treatment. We lay a great emphasis on animal well-being and husbandry. To avoid infections, immunocompromised mice will be kept under strict sterile conditions e.g. in individually ventilated cages with sterilised food and bedding. The cages will be changed in a dedicated animal transfer station and routine health screening will be conducted by the staff under the guidance of our named veterinarian.

Why can’t you use animals that are less sentient?

The project aims to study human prostate cancer and how it interacts with the rest of the body. This requires a model system that can form cancer and is similar to human biology (a mammal). This includes that they have similar organ structure, the ability to form cancer such as prostate cancer and have a complex immune system and comparable microbiome composition. Within the range of suitable models, mice are the least sentient species appropriate to conduct preclinical in vivo drug development studies and are widely used for this purpose. Although wild-type (normal) mice do not form prostate tumours spontaneously, genetic modification can trigger the formation of tumours that accurately resemble human disease. Unlike higher mammals, they can be kept relatively easily in a suitable and species-appropriate manner. Furthermore, their life cycle is relatively fast, so results can be achieved in shorter periods of time. Prostate cancer like most cancers is a disease of older age and needs time to develop. Studies in earlier stages are therefore not possible.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

During breeding and experimental planning: Experiments will be carefully planned to achieve maximum outcomes with the lowest amount of suffering. We will plan the breeding of animals accordingly. To reduce unnecessary breeding we will use already existing mouse mutant lines wherever possible. If an animal model with a novel genetically introduced mutation shows a phenotype that may cause discomfort or distress, we will genetically modify the line in a way that the mutation is induced (e. g. by using a drug) only if and where it is needed to address the specific scientific question.

We will plan procedures to minimise pain and distress and observe animals daily using trained staff. Immunocompromised mice will be kept under strict high barrier conditions eg in individually ventilated cages with sterilised food and bedding. Cages will be changed in a laminar flow cage change station using an aseptic technique. The animals are closely monitored, and tumour burden is kept within acceptable limits to minimise suffering. Animals will be checked daily by trained staff.

During surgery: We provide multimodal analgesia (painkiller) and anaesthesia during and after surgery and ensure the animal stays warm and hydrated during the procedures.

The animals are closely monitored, and tumour burden is kept within defined acceptable limits to minimise suffering. This will still allow us to address a given scientific question while reducing unwanted side effects.

During drug treatment: Before and throughout the treatment, tumour growth and animal well-being are monitored regularly. We will carefully choose drug doses that are effective but minimise side effects. When assessing treatment in mice for the first time, we will perform a small pilot study within a range that should not cause harm, to explore its possible side effects. Wherever possible, we will try to combine monitoring and treatment to reduce the overall number of stressful events for each mouse.

We will choose non-invasive methods to monitor tumour growth, which will reduce the number of mice needed. Imaging will also allow us to observe potential adverse effects earlier and end the study before the mice show clinical signs. Wherever possible, we will carry out monitoring and treatments at the same time to reduce stress and the number of separate interventions.

We will carefully choose drug doses that are effective but minimise side effects. For first time treatment in a specific mouse strain where side effects are yet unknown, we will conduct a small pilot study within a safe dose range based on prior results or data from others, to assess potential side effects. In this study, we will not use drugs with unknown toxicology.

At the end of each experiment, we will use the most humane methods of killing (euthanasia) possible.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Our research institute lays great emphasis on the optimisation of animal well-being. Both the researchers and BSU staff are attending regular conferences. We will make sure that we keep up to date with published guidelines such as the NCRI and ARRIVE guidelines. We work in close contact with the home office and ensure via internal training that every member of staff is up to date with their training.

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 them throughout the project, we will closely follow the resources provided by the National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs). These resources offer practical guidelines that help us ensure the humane use of animals in our research. Additionally, we adhere to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines when reporting our animal research. These guidelines are essential for transparently documenting our experimental procedures and results, ensuring that we provide sufficient detail to accurately represent our work. By staying up-to-date with these resources and guidelines, we can effectively incorporate advancements in the 3Rs principles into our research practices, ultimately promoting more ethical and scientifically rigorous animal experimentation.