



Home Office

Animals (Scientific Procedures) Act 1986

Non-technical summaries for project
licences granted April – June 2024 that
require a retrospective assessment



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1. Control of tissue repair by extracellular matrix

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

regenerative medicine, therapy, skin

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to better understand how cells the body protect themselves and recover from environmental damage or injury.

A retrospective assessment of these aims will be due by 9 October 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

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 research aims to unravel the body's mechanisms for self-protection and healing after injuries, identifying failures in these processes that lead to disease. This is highly relevant to human conditions including chronic wounds, which represent a significant socioeconomic burden due to prolonged healing and intensive care needs. This work strives to refine therapeutic strategies and enhance skin disease/wound treatment, ultimately easing the healthcare strain and improving patient outcomes.

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

This research is designed to dissect the body's intrinsic mechanisms for protection and recovery post- injury, identifying malfunctions that result in disease. Relevant to chronic wounds, a major socioeconomic challenge due to extended recovery periods and extensive care demands, the outputs are twofold:

Academic Benefits:

- Provide a clearer understanding of the body's self-healing processes.
- Identify specific points of failure that lead to prolonged wound healing and disease.

Patient Benefits:

- Inform the development of more effective therapeutic strategies.
- Improve disease management and treatment outcomes.
- Ultimately, this work aims to alleviate the burden on healthcare systems and enhance the quality of life for patients.

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

Scientists are anticipated to gain immediate to short-term benefits from a deeper mechanistic comprehension of pathophysiological processes. In the medium term, clinicians and patients are expected to experience the advantages of novel or enhanced products and treatment strategies. Over the long term, the general public will benefit from more effective disease treatments, contributing to a reduced economic burden on the NHS.

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

All results will be published open-access, discussed at international scientific meetings and with clinicians. Collaborations with clinicians and other researchers, including in industry/biotech will maximize the potential for these outputs to be translated into better treatment strategies that will directly improve patients' lives.

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.

Young adult mice will be used, specifically around 10 weeks old, due to their full development and stable hair cycle, ensuring consistent skin analysis. This age, and up to 25-30 weeks of age, is also useful for breeding.

Mice are the preferred model organism because of their significant genetic and physiological similarity to humans, enhancing the applicability of our findings to human health. Their genetic malleability allows for precise disease modeling, providing a unique in vivo perspective that captures the complex biological interplay involved in skin disease and tissue repair, something that in vitro, ex vivo, or computer models cannot fully replicate (e.g. a fully functional immune system).

In essence, young adult mice are crucial for a comprehensive understanding of healing mechanisms, offering invaluable insights that are translatable to human medicine.

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

Some animals in this project will be used for breeding, while other animals will be used for minor surgeries, where a small biopsy of skin is removed from the back skin of the mouse as a way of examining healing. The duration of surgery (biopsy) is only a few minutes, and takes place under full anaesthesia/anaelgesia. Mice recover fully and are monitored closely. The number of these procedures a mouse experiences is limited to 1.

A small number of genetically modified animals will be born of the Col1a2Cre-caNrf2 genotype and may be smaller than their littermates, with some of them experiencing a temporary hind-limb paralysis that begins in the first week or so and typically resolves itself by weaning age. However in some cases, this phenotype does not resolve sufficiently and leads to the inability of the mouse to thrive to weaning age. These Col1a2Cre-caNrf2 mice are classified as severe and are euthanized for humane reasons. The exact reason these mice experience this phenotype is not known, but it is related to their genotype. After weaning, Col1a2Cre-caNrf2 mice are still slightly smaller but show no obvious signs of discomfort and behave normally.

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

Adverse affects are expected to be rare for mice simply used for breeding. The genetically modified lines, when bred, do not often show adverse effects, although occasionally some genetic modified mice of the Col1a2Cre-caNrf2 genotype are smaller. In case the Col1a2Cre-caNrf2 mice, they can develop temporary hind-limb paralysis that prevents their ability to thrive to weaning age, they will be classified as severe and euthanised for humane reasons. Other mice with only mild/moderate paralysis will be monitored closely, and if old enough, be given food and water gel in the cage floor. However as mentioned, by weaning age this phenotype typically resolves itself. As adults, these mice, even if smaller, act normal, and do not display obvious adverse effects. After surgery (only on 10 week old adults), all mice tend to lose a small amount of weight which is typical. This is limited to a day or two after the surgery. In my experience I have never seen any adverse effects in Col1a2Cre-caNrf2 mice as adults following surgery that are any different than littermate controls or wild-type 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)?

<5% severe, 25-30% moderate and 70% sub-threshold.

What will happen to animals at the end of this project?

- Killed
- Kept alive

A retrospective assessment of these predicted harms will be due by 9 October 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

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 fully grasp the intricate mechanisms we are studying, it is essential to observe cells within a living mammal. Cells demonstrate unique behaviours in an in vivo context that cannot be replicated when cultured in a dish. Processes such as tissue repair and healing involves extremely complex interactions among various cell types, immune responses, and systemic factors, all essential to the process and can only be properly studied in a living organism.

While in vitro or ex vivo models provide valuable insights, and can reduce the number of animals needed for certain research questions, they fall short of capturing the full spectrum of biological responses present in a living system. Mice, with their close genetic and physiological parallels to humans, offer a more accurate representation of human conditions, ensuring the relevance and applicability of our findings to human medicine, particularly in advancing therapeutic strategies for tissue repair and skin disease.

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

Cell culture models have been used and considered. For example, scratch assays in cell culture can be used to simulate an "injury". These models involve cells grown until they fill a dish, and are then "scratched" with a plastic tip to create a "wound" or gap. The cells then grow and migrate to close the "wound". In reality however, this measures a cell's ability to migrate on a 2D plastic surface and does not really investigate "healing" aside from that specific aspect (migration) in an artificial context, with no input from other cell types or influence from a functional immune response. While this model can still be useful - the information gained is clearly limited.

Computer models are also useful for addressing certain specific questions, however there is no computer based model that can be used to simulate the wound healing process in its entirety.

Why were they not suitable?



While these models can be helpful to address some questions, their usefulness is limited since they do not provide an actual model for how cells behave in the context of a living mammal, where they are influenced by the surrounding 3D architecture, signals from other cells etc. Other models are also not able to take into account the impact of the immune system/response, which is a critical component of all tissue repair processes.

A retrospective assessment of replacement will be due by 9 October 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your experience?

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?

Mice will be housed together and only bred when the colony requires refreshing. As such, only one cage of males and one cage of females are required at a time for each type of genetically modified line. Five mice are able to be housed in each cage, and 4 genetically modified colonies are needed. 2- 3 breedings are needed per year. Additional mice are required for surgeries and numbers required are calculated using statistical methods.

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

Literature searches for the optimal age for breeding, how long the mice can be housed together prior to setting up a breeding etc. This allows mice to be housed for the maximum time before breeding is required, thereby reducing the number of mice in total generated/used. Power calculations allow for the determination of the minimum number of mice needed for the results to be meaningful. The NC3R's Experimental Design Assistant is used along with computer software (e.g. GPower) to help with determining sample sizes etc. Literature searches also show that multiple small biopsies taken from the same mouse can be analysed independently, as separate data points, without causing any additional discomfort to the animal. This greatly reduces the number of animals needed to achieve the required sample sizes.

To further reduce the number of mice utilized in this study, we will implement stringent standardization measures across all aspects of our experiment, including the environment, procedures, and animal handling. This includes the time of day procedures are carried out to account for circadian rhythm.

Consistent and controlled conditions will help minimize variability in our data, enhancing the reliability of our results and potentially allowing us to achieve statistically meaningful results with fewer animals. Animals whose genotypes are not suitable for experiments will



not be wasted but will have their tissues used by other researchers as part of the University's tissue sharing program.

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, cages. Males will be removed from breeding cages when sufficient mice are born, to reduce the number of new, unneeded pregnancies/litters. Pilot studies using cells in culture are used to generate as much data and justification possible before studies in mice, to ensure the usefulness of using animals to address the research question. When animal use is needed, pilot studies using a small number of mice will ensure that future procedures will be optimized and reduce the chances of any larger experimental procedure needing to be repeated due to technical mistakes or unforeseen complications.

A retrospective assessment of reduction will be due by 9 October 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

Refinement

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

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

Mice are the selected model for studying cellular responses to skin injuries due to their genetic and physiological similarities to humans, which enhances the ability of our findings to be relevant and translatable. In this study, we will induce a minor "injury" through a small biopsy, ensuring the wound size is minimal to reduce any potential discomfort to the mice. This technique does not necessitate invasive surgery or sutures, but is limited to the back skin, further minimizing pain and stress. To ensure the welfare of the mice, we will employ appropriate anaesthesia and anaesthesia during the procedure and provide appropriate post-procedural care, including pain management and close monitoring. Mice are also kept warm at all times while asleep, using appropriate heating pads during surgery. During recovery, mice will be kept in a warm recovery chamber with their own bedding which will result in familiar scents and reduce stress. Soft food will also be offered during the recovery period to encourage eating and aid recovery. The skin serves as an optimal organ for these investigations, given its accessibility and the straightforward nature of the procedure, aligning with our commitment to the 3Rs principle in animal research.

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

Mammals are required for this project to mimic humans more than less sentient animals would. Any potential discovery in other animals (e.g. flies), while potentially informative,



would require validation in mammals prior to being applied to humans for the purposes of addressing skin disease or non-healing wounds.

Among the appropriate mammalian models to study tissue repair, mice are among the least sentient compared to rats and other larger rodents, pigs, or primates.

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

Animals will be closely and routinely monitored, particularly during pregnancy, immediately after birth, before and after surgery. Pain will be minimized by using appropriate anaesthesia and analgesia as part of the surgical procedure, and mice will be allowed to recover from anaesthesia in a warm recovery chamber containing their own bedding to maintain a healthy body temperature and provide familiar scents, which reduces stress.

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

The PREPARE guidelines will be followed. Previously published work successfully using these procedures will also be used to guide the experimental design.

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, sign up to the NC3Rs newsletter, and attend Regional 3Rs symposium. To ensure continual integration of the 3Rs advancements throughout the project, we actively engage in ongoing literature reviews and participate in workshops and conferences focused on animal welfare. Our team will also partake in training programs to enhance our skills and knowledge in implementing humane practices. Collaboration with research groups renowned for their 3Rs commitment will further enrich our approach. Online resources, such as those provided by the NC3Rs, will be utilized for additional guidance and tools. Regular internal reviews will be conducted to assess and align our practices with the latest in 3Rs advancements, and we will maintain an open channel for team members and collaborators to suggest improvements, ensuring that we uphold the highest standards of animal welfare.

A retrospective assessment of refinement will be due by 9 October 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?



2. Gene regulation of cardiovascular disease 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
 - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

VEGF (Vascular Endothelial Growth Factor), Atherosclerosis, Cardiovascular repair and regeneration, Cardiovascular Development, Angiogenesis

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

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

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 find out more about how our blood vessels and hearts work, and what goes wrong when diseases happen. Our goal is to find new treatments, such as gene or cell therapy, or find drugs that can help fix these problems.

A retrospective assessment of these aims will be due by 3 October 2029



The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

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?

Coronary artery disease is a major cause of death and ongoing health issues for adults worldwide. It primarily occurs when arteries in the heart and brain become blocked, usually due to a combination of factors like atherosclerosis (plaque buildup) and thrombosis (blood clots). Understanding how cells and molecules contribute to plaque formation is crucial for developing treatments to prevent artery narrowing and blockage. Our goal is to study the role of specific molecules in atherosclerosis development within different types of cells in the plaque, such as macrophages, smooth muscle cells, and endothelial cells. Our previous research has shown that specific receptors to a growth factor are involved in atherosclerosis and could be potential targets for therapy. We aim to further understand how they affect plaque build-up using knockout animals or administering therapeutic molecules.

After a heart attack, mammals experience irreversible loss of cardiac muscle, resulting in a permanent scar that impairs heart function. However, in zebrafish, a temporary scar forms after injury, which is gradually replaced by new, functional heart tissue. Studying zebrafish has been crucial for identifying the molecular pathways involved in this regeneration process. Previous research suggests that our receptors of interest also play a role in cardiac regeneration. We plan to continue investigating this by using zebrafish with altered gene expression to understand how those receptors influence the different stages of heart regeneration. This will help us uncover the specific cellular functions modulated by these receptors during the regeneration process.

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

This project aims to take what we have learned in the lab and investigate if it can be applied in vivo to study heart and blood vessel disease similar to those seen in humans. We want to find out if changing the expression of certain genes or blocking specific signals can protect against heart and blood vessel diseases. By studying mice and zebrafish, which have similar biological processes to humans, we hope to see if our ideas could eventually help patients.

We are particularly interested in looking at how changing the activity of certain genes and signals affects heart and blood vessel diseases. This could give us clues about new treatments for conditions like heart attacks and heart failure. We will also explore ways to reduce the buildup of artery plaque build-up and help the heart heal better after a heart attack.

If our experiments go well, we hope to publish our findings in a top scientific journal. This could pave the way for new treatments that might help people with heart and vascular diseases in the future.

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



Findings from this work will be made available to other scientists through publication in peer-reviewed journals. We will increase the shared knowledge of the scientific community in the fields of vascular diseases and regenerative biology. Under the previous project we published 3 papers (and 3 more are currently in preparation). Because we conduct basic research, clinical translation is further down the line however our work is essential to identify molecules and their receptors/effectors important for cellular signalling driving diseases and/or regeneration.

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

We will disseminate our research by attending and organising conferences/seminars nationally and internationally. Under the previous project, we presented our findings at 2 international and 2 national scientific meetings (2019-2023) and I was the co-organiser of a 2-day workshop focused on animal models of regeneration. Our field of research is vibrant and highly collaborative and we share resources effectively with one another.

Species and numbers of animals expected to be used

- Zebra fish (*Danio rerio*): 10,000
- Mice: 2,000

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 how vascular disease start or worsen, using special mice and fish that have been changed at the genetic level. These animals either have certain receptors or molecules related to blood vessel growth turned on or off. We might also give them drugs that affect the signalling of these receptors. Then, we put them on a high-fat diet to see how it affects plaque build-up in their arteries, which can cause vascular disease.

Vascular disease is complicated, involving different types of cells, and depends on the diet the animal eats. Among animal models, mice are the best for studying how artery blockages happen, and there isn't a better model available.

For studying how tissues repair themselves, we use genetically modified zebrafish that are good at healing naturally. Zebrafish are perfect for studying how the heart can regrow because the complexity of the cardiac tissue is very similar to the mammalian heart. Additionally, the cellular processes stimulating the growth of new blood vessels are comparable to those activated in human tissue repair.

We can even track specific cells using genetically modified fish that express fluorescence in certain types of cells, making it easier to understand how particular cell types behave during regeneration.

When it comes to studying how blood vessels repair themselves, we use zebrafish embryos. We create a small injury to their blood vessels with a needle, and then we observe them as they heal over the next few days. They are transparent and easily



observable under the microscope, allowing us to track how vascular cells proliferate and heal in real time.

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

- To study the development/regression of vascular disease, genetically modified mouse models will be fed a diet high in fat to induce the build-up of plaque. Depending on the model, the loss of expression of specific genes can either be constitutive (our preferred option) or inducible. The latter option will be chosen if the loss of the gene is detrimental during development and must be delayed to minimise the suffering of the mouse. This is typically done by injections or oral administration, which will be refined and kept to a minimal number of occurrences. The mice will not be subjected to any surgical intervention.
- To study heart regeneration, we routinely use the cryoinjury model of cardiac myocardial infarction. To do so, we briefly anaesthetise the fish and apply a probe made up of a fine metal filament that has been cooled down in liquid nitrogen. The probe is in contact with the cardiac tissue only for a few seconds and the fish is then placed back into its tank, without the need for suturing. The overall surgical intervention does not last more than 5 minutes and the fish resumes swimming after less than a minute. No further procedures are necessary until the end of the experiment.
- Vascular regeneration is investigated following caudal fin amputation, which consist of cutting up to 50% of the fin. Similarly, the fish is under anaesthesia only for a few minutes and resume swimming shortly after being placed back in its tank. The fish might be anaesthetised again to quantify the size of the regenerated tissue but no further surgical intervention will be conducted until the end of the experiment.
- To study vascular repair, we use zebrafish embryos which are briefly anaesthetised to restrict their movement. It is to note, that, contrary to adult fish, the embryos do not rely on gills for breathing as oxygen freely diffuses through their skin. With the discrete prick of a fine needle, we disrupt the vascular network of the embryos. They are then placed back to water without anaesthetics and regain normal activity quickly. We then observe the subsequent repair by observing them under the microscope, for which they must be anaesthetised again, however, no further surgical intervention will be conducted until the end of the experiment.

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

- Few adverse effects (e.g., skin irritation) are expected from the atherosclerosis study (plaque build-up in the arteries) as the mice are not subjected to any surgical intervention. The mouse genetic models we are planning to use are not known to have adverse effects. Atherogenic diets are expected to increase body weight.
- Fish undergoing cardiac cryoinjury will feel only transient moderate to severe pain, which may be managed with the administration of analgesics. The fish resume swimming shortly after the surgical intervention and previous behavioural monitoring indicates that most fish experience only moderate pain. However, it is still to be determined which analgesic agent is best to alleviate the effects of the surgery on the fish.



- Fish undergoing fin amputation will experience mild to moderate pain which may be managed with the administration of analgesic agents. No other detrimental effects have ever been observed in the past or are expected.
- The damage inflicted by the needle to the vascular network of the embryo has little observable detrimental effect. The damaged tissue starts the healing process almost immediately. The embryos are closely monitored, and previous experience showed high survival rate. Nevertheless, it is unknown and currently debated if the zebrafish embryos experience pain at this early stage of development. Repeated anaesthesia at later developmental stages would induce mild distress.

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)?

- Vascular disease/ atherosclerosis study (mouse model): maximum expected severity will be moderate and only attained if unexpected adverse effects occur, such as skin irritation due to the diet. However, most of the animals under this protocol, will be classed under mild severity.
- Heart regeneration study (Adult Zebrafish): 90% of fish under this protocol are expected to experience moderate severity, 10% of fish might experience transient severe pain due to lack of efficient analgesic cover.
- Fin regeneration study (Adult Zebrafish): 90% of fish under this protocol are expected to experience mild severity, 10% of fish might experience transient moderate pain due to lack of efficient analgesic cover.
- Vascular repair study (Zebrafish Embryos): As stated earlier, it is difficult to ascertain pain in this model. However, because of the invasive nature of the injury which will repair over the following period, a maximum severity to be described as moderate is to be expected.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

A retrospective assessment of these predicted harms will be due by 3 October 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

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 learned a lot from studying cells in dishes about how our growth factors and corresponding receptors of interest work together. Now, we need to check if what we found in those experiments holds true in living organisms. In all the protocols used, alternatives are not available that replicate the response of, for example, the heart or a blood vessel, to injury, or that reproduces the complex cellular environment of a live tissue during repair.

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

If cell culture/co-culture experiments are useful to investigate up to a couple of cell types simultaneously and helpful to dissect and investigate specific signalling pathway by silencing gene expression or chemical inhibition, the build-up of plaque in arteries results from the interaction of several cell types, including the circulatory immune system. However, when possible, we will use alternative cell culture and ex vivo models as much as possible for pilot work preliminary to animal studies, and for more extensive analyses and dissection of signalling mechanisms.

Why were they not suitable?

Cell culture and ex-vivo explants do not recapitulate the complex environment of the biological response to injury. For example, inflammation brought up by immune cells requires a fully functional circulatory system. Additionally, the complex microenvironment made up of extra-cellular matrix and factors secreted by several cell types and their finely orchestrated interactions are only possible in living organisms. Additionally, because we investigate disease progression and regeneration of full organs such as blood vessels or the heart, the time frame of the response is long and relies on a succession of timely orchestrated processes. For example, during zebrafish heart regeneration, all phases, from inflammatory phase (characterised by the primary immune response), the reparative phase (with the activation of the epicardium (the outer layer of the heart) and revascularisation of the heart) and finally the regenerative phase (proliferation of cardiac muscle cells) must occur sequentially to allow successful regeneration. Similarly, in vascular disease, the development of the plaque is dependent on several factors (including blood flow), cells types, and their secreted factors and a complex environment. Such complex environments cannot be modelled in vitro and using ex-vivo models.

A retrospective assessment of replacement will be due by 3 October 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your experience?

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 estimated those numbers on previous experience, my previous PPL and animal returns from the past 5 years. I have considerably reduced the number of animals as I have limited the number of protocols from my previous project licence.

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

I routinely use the NC3R's Experimental Design Assistant to design in-vivo studies when applying for grants. Although useful in understanding the final number of animals used for the experiment, the

NC3R's EDA unfortunately cannot predict or take into account the animals that are generated via breeding but are not of the correct genotype and therefore that cannot be used. However, if not useful for preliminary studies of my own, I systematically offer the carcasses of those animals to colleagues who need tissues for their own studies. In our institution, communication for sharing resources is efficient and facilitated by the animal technical team.

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

I have over 10 years of experience in breeding animals for research, including mice and zebrafish. I systematically and carefully monitor the number of breeding cages to manage our resources fully. We visit the animal units regularly and perform inventories to understand the breeding performance and monitor the fertility of our experimental animals. Additionally, we genotype our animals to ensure the correct breeding are in place and to minimise the number of animals generated. For zebrafish, homozygous lines are kept to produce experimental animals and age-matched wild-type animals are used as control. When possible, and to minimise genetic drift, homozygous lines are outcrossed yearly.

If no data can be extracted from the literature, pilot studies are performed on a restricted number of animals. This allows us to understand the amplitude of the effect of a specific treatment, and informs us on the number of total animals required to attain sufficient power for our experiment.

Finally, as mentioned above, the sharing of tissues is highly facilitated by excellent communication between colleagues and the animal technical team.

A retrospective assessment of reduction will be due by 3 October 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

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 model of atherosclerosis (blood vessel plaque build-up) is very well refined and has been used extensively in the field. The mice are not expected to be in any pain during the whole timescale of the experiment. They are left to exhibit normal behaviour and care is taken to enrich their environment to minimise harm.

From experience and informal discussions with colleagues using similar models, the heart injury model is known to be tolerated by adult zebrafish. They experience a transient level of pain which can be managed via the administration of analgesics, so it does not exceed moderate severity; however the best analgesics regiment is yet to be fully defined, therefore a few animals might experience transient severe pain if the analgesia coverage is not fully efficient.

Similarly, from experience, the zebrafish fin amputation model is well tolerated and no adverse effects exceeding mild discomfort have been observed to date with analgesics administration.

Finally, the needle injury model to induce vascular repair in the zebrafish embryo is using the less sentient and more immature life stage. Although debatable if the embryos feel any pain at all, injuries are well tolerated, and embryos continue their development normally whilst repairing the wound. With this new PPL application, we wish to observe the repair process fully which includes the recruitment of immune cells but also the subsequent growth and reorganisation of blood vessels on a longer timeframe.

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

The development of plaque build-up to investigate vascular disease relies on a fully functional and a complex immune system, as well as a timeframe that is not compatible with less mature life stages. In fact, the mice must be specifically raised on a pro-atherosclerotic genetic background, as animals, in general, do not develop plaque naturally.

To investigate regeneration, animals must recover from the injury to allow the regeneration process to happen. For the zebrafish cardiac injury model, this occurs over a 90 days period following the surgery, and for the fin regeneration model, this is achieved over a 21 day period. Similarly, they rely on the interaction of several cell types, their secreted molecules and the deposition of a complex microenvironment that cannot be mimicked by less mature, less sentient life forms.

Similarly, bearing in mind the needle injury is carried out between 3- and 4-days post-fertilisation, the subsequent inflammation and vascular repair occur over a period that exceeds the time during which the zebrafish embryo is not protected under the Animals (Scientific Procedures) Act 1986 (ASPA).

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

With the help of our animal technical team, we implement rigorous monitoring of our animals, both in routine husbandry but also during post-operative care. All zebrafish



surgeries occur in the morning to allow a long recovery period with staff on site to ensure the level of severity described in the project licence is not exceeded.

Additionally, as mentioned previously, we also aim to investigate what is the best analgesic regimen for the recovery of our zebrafish protocols, as to date, the standardised use of analgesic agents has not been demonstrated to efficiently alleviate pain for all our models.

Finally, when administering drugs in mice we currently use intra-peritoneal (IP) injections. When/if possible, we will aim to administer drugs via oral gavage, although, it is again debatable if IP injections are more or less stressful than oral gavage for the mouse.

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

I will consult published guidelines to assist with planning animal research and testing, such as the PREPARE guidelines:

<http://journals.sagepub.com/doi/full/10.1177/0023677217724823>

Other resources 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?

I stay informed on the published literature in animal welfare. In particular, we receive regular updates from our animal technical team and the Animal Welfare and Ethical Review Body (AWERB) about recent advancements in refining in-vivo methods and the 3Rs (Replacement, Reduction and Refinement). I regularly attend relevant seminars and conferences where information on animal welfare is disseminated (RSPCA meetings, online International Zebrafish Society (IZFS) seminars etc...)

Additionally, being a member of the relevant societies and attending local seminars, I have access to a wide range of resources and a network of colleagues working in the field, available for informal discussion and advice on best practice.

In our institution, we are very lucky to have access to a dedicated team of veterinarian professionals who are world-leading experts in animal ethics and welfare. I have been in regular contact with our Named Veterinary Surgeon (NVS) to refine our protocols and will continue to do so for this project. We also receive regular seminars organised by our the NC3Rs Regional Programme Manager and our lab receive the NC3Rs newsletter. Finally, our animal unit management team offer regular PPL refresher courses and our training records are regularly checked to ensure our practical skills are up to date (e.g., Schedule 1 and era-notching/fin-clipping are regularly re-assessed).

A retrospective assessment of refinement will be due by 3 October 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?



3. Inflammatory mechanisms in cardiovascular 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 word

Myocardial infarction, Myocarditis, Heart failure, Inflammation, Cardiac remodelling

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

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

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 further our understanding of the inflammatory response, the body's response to harmful stimuli that is largely mediated by white blood (immune) cells, to heart- and blood vessel-related (cardiovascular) disease. This project aims to identify key mechanisms involved in cardiovascular diseases that have in common a significant inflammatory component. These include heart attack (myocardial infarction, where the blood supply to the heart muscle, or myocardium, is occluded) and myocarditis (a condition characterised by inflammation of the heart muscle, which is most frequently a result of viral infection or the body's response to infection).



A retrospective assessment of these aims will be due by 3 October 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

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 relevance of the cardiovascular system for human health is highlighted by global death statistics: despite modern treatments, almost a third of all deaths are caused by cardiovascular diseases such as heart attacks. Inflammation plays a central role in many cardiovascular conditions, but no anti-inflammatory treatments are routinely used. Thus, investigating immune and inflammatory mechanisms involved in cardiovascular diseases provides an opportunity to identify new treatments that could have a huge impact on public health.

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

This work is expected to significantly increase biological and disease-related knowledge that will be highly relevant to the treatment of heart-related disease. Specifically, the project should substantially increase our understanding of the inflammatory mechanisms that are critical in the early response to cardiovascular disease, including myocardial infarction and myocarditis, and subsequent progression to heart failure. Following these insults, the heart can undergo a complex process of decline referred to as (maladaptive) 'cardiac remodelling', which involves deterioration at molecular, cellular, and whole-organ levels, leading to heart dysfunction and ultimately heart failure. In some patients with heart failure, maladaptive remodelling can reverse back towards normal – termed (adaptive) 'reverse remodelling'. We aim to identify specific drivers of adaptive versus maladaptive cardiac remodelling, as well as pathways that promote reverse remodelling, which we anticipate will result in high impact publications.

By elucidating underlying mechanisms and by undertaking experimental studies in vivo, this research may provide the basis for devising novel therapeutic strategies for human cardiovascular disease.

As our projects have a strong translational aim (e.g., to develop new drugs and treatments for cardiovascular disease), we already have ties, and plan to expand them in the future, with pharmaceutical companies for the subsequent development of the therapeutic leads we identify.

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



This project, investigating the regulation of inflammation in cardiovascular disease, will have several beneficiaries, including the participants, collaborators, and the contributing institution.

Moreover, researchers in the fields of heart failure, heart attack, myocarditis, and inflammation are expected to benefit directly from the knowledge gained and disseminated from this project.

New knowledge from this project could be applied widely across any discipline interested in the immune response to injury, which is an enormous field both within the UK and elsewhere.

Overall, this work is expected to have significant, international impact, both within cardiovascular science and in other disciplines interested in the immune and inflammatory response to tissue injury.

Finally, a significant focus of the project is to identify new treatments targets and potential therapies. We therefore expect that, in the longer-term, this work will benefit patients with cardiovascular and other inflammatory diseases and will have wider public health impacts by reducing the consequences of these debilitating conditions.

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

Results obtained in this project will be published in open-access journals and will therefore add to the body of publicly available knowledge. Findings will also be disseminated to the scientific and medical community by presentations at seminars and conferences.

Our Centre has extensive national and international collaborations which will further enhance the sharing of results. We also host visits from other researchers for them to obtain direct exposure to our work.

Species and numbers of animals expected to be used

- Mice: 14,900

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 study will be performed using mice because:

- (1) What we learn about the inflammatory response to heart disease in mice can help us understand how it works in humans, as many key pathways are the same.



- (2) Methods to give mice heart disease are already available for the study of human disease. It is also already possible to measure important heart-related outcomes in mice, such as cardiac size and function.
- (3) We can alter mice's genes to study very specific pathways involved in heart disease. This allows the impact of a particular gene to be examined in a far more specific manner than can be achieved with most drugs.
- (4) We have extensive experience of working with mice, including those with changed genes, to learn about heart disease.

Most work will be undertaken using adult mice because the immune system can change in older mice, which might confuse our results. Also, we have a lot of experience of studying heart disease in adult mice.

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

We will induce heart disease, using either heart attack (myocardial infarction) or myocarditis. Genetically altered animals (GAAs) will be used to investigate the effects of specific proteins and pathways of interest. Heart function will be monitored non-invasively. Sometimes, we will give them chemicals or drugs to help us understand how inflammation contributes to heart disease, and to test new ways to treat heart diseases. Further studies will be performed after killing the animal. Typically, animals are followed up for a maximum of 3 months. The number of procedures will be kept to the minimum necessary to pursue the aims of the project.

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

In general, the expected impacts are the development of heart failure and those of the experimental treatments. A small proportion of animals may experience problems soon after the operation. If this happens, we will treat them quickly or cull them, so that the duration is expected to be <1 day. The animals might have changes in blood pressure, breathing, and heart function, and they could feel pain or stress. In rare cases, if any animal has a severe problem, including their heart beating abnormally, we will promptly manage it as outlined under the individual protocols or will be culled so that the duration of such effects is expected to be <1 day. A small percentage of animals used for experimental heart attack (myocardial infarction) or myocarditis may develop sudden onset heart failure or heart rhythm problems, but this is only expected in up to 10%.

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

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



Animals in protocols with no surgery will experience a mild to moderate severity (e.g., characterisation of GA and WT mice). Most animals in surgical protocols will have a moderate severity, as factors such as pain relief and good aseptic technique will mitigate against severe adverse effects. These animals will be closely monitored and additional provisions such as supplemental heat, access to food and water and painkillers will be provided to minimise adverse effects. The experimental procedure of inducing heart attack (myocardial infarction) is associated with a peri-operative mortality of up to 10%. Most, but not all, animals that will exhibit adverse effects are expected to be identified during the surgical operation whilst under anaesthesia and will therefore not be recovered but instead culled under anaesthesia. In addition, the predicted mortality due to heart failure post-surgery and during the animal's recovery phase is up to 10%, and animals that show signs of heart failure will be culled.

Additionally, in 10% of cases sudden death may occur.

The experimental procedure of inducing myocarditis (a condition characterised by inflammation of the heart muscle, which is most frequently a result of viral infection or the body's response to infection) is associated with acute or chronic heart failure in 10% of animals. Animals that show signs of heart failure will be culled. Alongside direct infection of the heart, infection of the pancreas also occurs. Such mice display reduced health status, which may include a raised respiratory rate, panting, reduced activity, reduced body weight, piloerection, hunched posture, pale colour of extremities and mucosa and a reduction in body weight. Severe effects, such as reduced activity, hunched posture, and piloerection, are anticipated in up to 30% of infected animals. Animals showing such severe effects will be culled.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

A retrospective assessment of these predicted harms will be due by 3 October 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

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?

When we study heart diseases in animals, like after a heart attack, the immune and inflammatory response is highly complex because it involves lots of different cells and



parts of the body, like the heart, bones, and spleen. These interactions can change blood pressure, heart structure, how blood moves, and how cells work or die. We can't study all these details just by looking at cells or organs by themselves – we need to see how everything works together in the animal's body. However, we will also do experiments in the laboratory and using computer simulations to guide our animal experiments and to help us understand them better.

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

We want to use human experiments whenever possible, including using human tissues and cells. We considered organoids and/or organs-on-chips and are working with others to use tiny heart models grown in labs (organoids) for high throughput screening of potential medicines and to test medicines. We also do tests with heart cells made from special stem cells (inducible pluripotent stem cells) to determine what to study next in animals, which helps us use fewer animals. Finally, we look at big datasets from other studies (including RNA-sequencing experiments) to learn as much as we can without using animals. We have considered other model organisms such as zebrafish.

Why were they not suitable?

In our heart disease studies, we need to see how different immune cells in the body, like those in the heart, spleen, and bones, work together. Cells in the heart are not available from patients because of the urgent nature of heart problems and risks in getting heart samples. Similarly, organoids, organs-on-chips and/or computer simulations are unable to fully show us how everything works together in the body, especially how cells interact. Therefore, animal models are the only avenue, at this stage, that can provide the necessary information.

Use of mammals like mice is essential, as other animals such as zebrafish are not suitable for experimental myocarditis or heart attack (myocardial infarction) as their hearts are too different from humans, it is not possible to induce heart attack in the same way (by blocking a heart artery), and they cannot be used to measure key physiological readouts such as blood pressure.

A retrospective assessment of replacement will be due by 3 October 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your experience?

Reduction

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



numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

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

The experimental design and analysis methods are based on careful consideration of statistics, power analyses and good laboratory practice, and have undergone stringent review as part of the grant-awarding process. Total numbers are also based on breeding considerations for gene-modified animals. For qualitative experiments (e.g., immunohistochemistry), the amount of material required will be the minimum necessary to provide an adequate description.

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

We have implemented heart imaging methods such as echocardiography and magnetic resonance imaging to non-invasively monitor heart structure and function. This helps us see how their hearts change over time with fewer animals needed. This also reduces variability by allowing comparisons at different time points in the same animal. Protocols will be written for each experiment as part of good laboratory practice, including treatments, the number of groups and number of animals/group (based on power calculations) and the outcome measures.

It is not always easy to estimate how many animals will be required to maintain a mouse line. We will measure production and breeding performance and ensure we only use as many as necessary.

At the end of the experiment, when animals are sacrificed, we have developed efficient protocols that ensure the maximum possible information can be obtained from each animal (e.g., protocols for blood and heart collection to allow RNA sequencing, flow cytometry and protein readouts such as Luminex all from one animal). This also significantly reduces the total numbers of animals required.

We will continue to take advantage of the experimental design tools to keep numbers low e.g., NC3Rs online advice resource portal (<https://www.nc3rs.org.uk/topic-specific-resources-0>). The experimental design assistant will also 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?

Breeding of experimental animals will be set up to ensure that litters contain both test and control animals to ensure that the least number of animals are bred for any specific experiment. Furthermore, we will use animals of both sexes to similarly ensure that as many animals as possible are used for experiments. For GAAs, where suitable lines already exist (established by searching databases), animals will be obtained from the relevant supplier. We will use any previous knowledge from our own studies or the



literature to avoid using more than the minimum number of animals. We will also use computer simulations to guide animal experiments and optimise animal numbers. For many studies, non-invasive techniques that allow serial assessment of cardiovascular function will be used, allowing reduced numbers of experimental animals. This is especially valuable when assessing the impact of medicines aimed at preventing or slowing the development of heart failure. If novel treatments are tested in animals, the literature will be reviewed, and the drug company will be contacted to ensure that the most up to date knowledge on drug dosage and administration is followed. A small pilot study will be used to determine the impact of any new drug on animal welfare before continuing to a fully powered study.

A retrospective assessment of reduction will be due by 3 October 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

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 of heart attack (myocardial infarction) and myocarditis (a condition characterised by inflammation of the heart muscle, which is most frequently a result of viral infection or the body's response to infection) will be used, which is justified as follows:

Choice of species

Wild type and genetically altered mice will be used in the project. In most cases, we do not anticipate baseline phenotypes with adverse effects. State-of-the-art imaging techniques, such as high frequency echocardiography and cardiac magnetic resonance imaging, are used to assess murine cardiovascular structure and function in an analogous manner to humans. These are both available and characterised in mice as well as being non-invasive, thereby minimising pain, suffering, distress, or lasting harm.

Choice of models and methods

The models are chosen to provide high quality information about key heart changes in diseases like heart attacks and inflammation of the heart. These include heart structure,



function, cell function, and inflammation. The models mimic the major causes of inflammatory cardiovascular disease in humans and are all well established and validated in the published literature.

For studying heart attacks, we have implemented non-invasive intubation techniques, non-invasive ways to estimate heart attack size, and other components of remodelling like elastin, using magnetic resonance imaging, 3-dimensional echocardiography to monitor changes over time in cardiac function, and non-terminal measurement of heart damage using a blood test.

For myocarditis, we will use a scoring system to define monitoring intervals and allow early identification of mice prior to reaching humane endpoints. The use of a virus is preferable to models of autoimmune myocarditis (where antibodies directed against the heart are used), which are challenging to induce and require transgenic mice that have high mortality.

The methods to be used to obtain in vivo experimental measures are the most refined available for the assessment of cardiovascular structure and function in mice. Specifically:

- The use of intravital microscopy to study inflammation in the cremaster muscle allows valuable insight into the inflammatory process, without performing myocardial infarction, in initial studies.
- We use state-of-the-art, non-invasive echocardiography and imaging methods, including serial assessment.
- Haemodynamic assessment performed as a terminal procedure uses gold-standard pressure- volume analysis methodology.

To study how inflammation contributes to heart disease, and to develop new treatments, like medicines and cell therapies, some animals may undergo adoptive transfer of cells or bone marrow transplantation, with or without prior irradiation, to change how their inflammatory response works. This might include xenotransplant of human haematopoietic stem and progenitor cells, and/or adoptive transfer of cells, including engineered cells. Mice that do not require irradiation will be used where possible. The general approach proposed in this programme is well established in the literature (and in our previous work) and has been shown to yield clinically relevant information.

Severity classification protocols and measures to minimise suffering

We do not anticipate major detrimental effects of genetic alterations in the mice. Animals exhibiting any unexpected effects that may lead to pain, distress, suffering or lasting harm will be managed in consultation with the Named Veterinary Surgeon. In the case of individual animals of special scientific interest, advice will be promptly sought from the local Home Office Inspector.



Protocols 3 and 4, which involve cardiac remodelling and heart failure following myocardial infarction (heart attack) and myocarditis (a condition characterised by inflammation of the heart muscle, which is most frequently a result of viral infection or the body's response to infection), respectively, have severe classifications.

All surgeries will be conducted under aseptic technique, and with medicine to reduce pain, by trained staff. After experiments, we will deliver the highest levels of care, including additional monitoring and discussing with the Named Veterinary Surgeon if required.

As most side-effects in Protocol 3 is in the first 24 hours after surgery, or after 4 days in Protocol 4, animals will be closely monitored at frequent intervals during these periods. Animals will be reviewed at the end of the working day on the day of surgery and any that are considered likely to die overnight will be killed by Schedule 1 methods. Continued post-procedural support when required will consist of the use of heat, analgesia, hydration (water jelly), wet mash as necessary.

After surgery, an animal might develop a wound infection, but this is rare. Any animal showing wound swelling, redness, or discharge, but is otherwise well, may be treated on advice of the Named Veterinary Surgeon. The animal will be killed if no improvement is seen in the first 24 hours of treatment or if its condition deteriorates before then. If a wound opens again, we might close it once more, but only within two days of the first surgery. Where absorbable skin sutures are used, sutures will not be removed unless absorption is incomplete and seems problematic for the animals, after 14 days; in this very rare instance light inhalational anaesthesia may be used for restraint where usual methods of restraint are likely to cause undue stress. We will work closely with the Named Veterinary Surgeon and will seek advice on animals whose welfare is giving cause for concern.

Following myocardial infarction or myocarditis, animals can develop heart failure. We will monitor them carefully for several weeks and any that are in a poor clinical condition will be treated in consultation with the Named Veterinary Surgeon and culled within 24 hours if there is no improvement or if their condition deteriorates/human endpoint is reached before then. Signs may include loss of weight, listlessness, and rapid breathing in the late stages, which will be closely and regularly checked and monitored during the study. However, animals will not be allowed to progress to late-stage heart failure. Any animal showing signs of heart failure such as listlessness and rapid breathing will be humanely killed. Any clinical problems will be dealt with in consultation with the Named Veterinary Surgeon.

Animals will be humanely killed at a pre-determined endpoint or at the end of the study, whichever happens first.

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

The development of cardiac remodelling and heart failure is a chronic process that is not possible to achieve in the short time span of a terminally anaesthetised animal. We need to use mammals which closely represent the human condition and the complex



interactions that occur between inflammatory cells and organ systems. Other animals such as zebrafish are not suitable for experimental myocarditis or heart attack (myocardial infarction) as their hearts are too different from humans, it is not possible to induce heart attack in the same way (by blocking a heart artery), and they cannot be used to measure key physiological readouts such as blood pressure. Another major advantage of using mice is the wide availability of genetically altered lines or the ease of their generation, allowing the impact of specific genes to be examined far more specifically than achievable with most pharmacological tools.

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

As the major component of morbidity and mortality are in the first 24 hours after surgery for myocardial infarction or the first 4 days following induction of myocarditis, animals will be closely monitored at frequent intervals during this period. Animals will be reviewed at the end of the working day on the day of surgery and any considered likely to die overnight will be euthanised. Any animal that is observed to be in pain or distress will receive analgesia via a predetermined regime agreed in advance with the Named Veterinary Surgeon along with other supportive measures including warmth and wet mash. In post-procedural recovery period, animals will be monitored at least twice daily. Careful attention will be paid to heating, analgesia, body weight, surgical wound-sites, hydration, and signs of pain or distress. The characteristics and treatments applied to all animals in these categories will be reviewed, and the Named Veterinary Surgeon involved where necessary, to identify and institute possible refinements, including to anaesthesia, surgical techniques, doses, and analgesia.

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

The NC3R's publishes several resources, which will be employed, including:

- ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines.
- EDA (Experimental Design Assistant)
- Tech3R's for those carrying out regulated procedures and animal handling.
- PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines.
- LASA Good Practice Guidelines on Administration of Substances (techniques for dosing).
- Home Office Animals (Scientific Procedures) Act 1986 (ASPAs) and Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific



Purposes (published 2014) are used to ensure legal compliance with the Standard Conditions of all license holders and animal users.

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

All researchers and animal users will be kept up to date on advances by journal reviews, reading published information or updates, systematic review of processes with implementation of any appropriate refinements or improvements where possible and required, and by following updates from the Named Information Officer. Training and additional resources will be made available to all staff throughout the project. We will monitor the NC3R's website, receive guidance from AWERB and keep up to date with published literature. Good communication will ensure cascade of information to PIL holders, this will include discussion of the 3Rs with colleagues.

A retrospective assessment of refinement will be due by 3 October 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?



4. Regulatory Testing of Biological Toxins and Antitoxins

Project duration

5 years 0 months

Project purpose

- 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

Botulinum, Toxin, Potency, Assay, Antitoxin

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

Contains severe procedures

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 licence authorises the conduct of testing procedures to ensure the safety, efficacy, stability and overall quality of toxins and associated proteins used for medicinal products in accordance to registered marketing authorisations held with national and international regulators and in accordance with Good Manufacturing Practice. This is to aid in the development of medicines and to provide mandatory information to regulatory authorities to allow marketing approval.

To provide testing services to assist with product development, improvement and clinical trials associated with toxins and associated proteins.



A retrospective assessment of these aims will be due by 12 October 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

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?

Governments require that substances used for medicinal purposes are safe and that their potency before administration is deemed accurate. The work detailed in this project will allow the continued safe development, production and use of Botulinum Toxins and their derived products for the treatment of a wide range of medical conditions.

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

The overall safety of medicinal products (drug products) is paramount for the consumer. These products must be fit for the intended use and therefore the overall efficacy and quality must be assured. During the development of the active drug substance and subsequent formulations, safety and efficacy must be demonstrated and the product must be monitored to confirm that no changes have occurred that would affect its quality so that when the product is made available to the consumer their safety is ensured.

The work detailed in this project will allow the continued safe development, production and use of biological toxins and their derived products for the treatment of a wide range of medical conditions. The product on this licence has been licenced across a range of disorders including, idiopathic cervical dystonia, axillar hyperhidrosis (excessive sweating) and spasticity in cerebral palsy.

Legislation prohibits the marketing of drug products without determining the potency which will be detailed in the relevant marketing authorisations. Testing is conducted to meet the requirements of existing marketing authorisations. The ability to test these substances ensures that products of known potency are used therapeutically, eliminating serious health risks to the end user. The testing of products in pre-clinical trials ensures patients in clinical trials are also appropriately protected and enables the development of new botulinum toxin products with more favourable characteristics for patient use. Any prevention of testing would have an immediate impact upon patients, preventing safe release of products and adverse medical consequences.

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



The continued safe development, production and use of biological toxin-derived products will benefit patients treated. The prevention of these tests would result in the release of these products being delayed or denied. Depending on the exact nature of the conditions being treated in patients this could result in serious health implications. Additionally, due to the large use of “off label products” and production in territories where testing may be less vigorously controlled, there is potential for the end user to be “treated” with less well-defined products.

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

We provide a service to clients and as such do not own the data generated nor have the right to publish the data. As an organisation working under confidentiality agreements, the clients will be provided with the data which they will independently use to inform their marketing/development processes.

Given the confidentiality of the work carried out on behalf of sponsors/customers it is difficult to engage in sharing of information at conferences, workshops, blogs or published literature.

Species and numbers of animals expected to be used

- Mice: 352,000

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.

Historically much of the work undertaken on toxin products has used mice, as they are regarded to be the lowest animal neurophysiological model considered appropriate for predicting what is likely to happen in humans. Adult mice are used because fully developed function is essential for the assays tested as part of this project.

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

Adult mice of an appropriate strain and weight range will be housed in a purpose-built facility in social groups. Animals will receive one intraperitoneal injection of no more than 0.5mL of product and then be returned to their cage. They will reside with other individuals for the duration of the test, but given the nature of the product, as the test progresses, animals will be removed and humanely killed should the onset symptoms be causing impact on the animals' welfare. Animals are observed every hour for the duration of the test. The animals are on test for 72-96 hours and on completion of the test will be humanely killed using a Schedule 1 method.



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

The effects of the product tested on the mice can result in the death of the animal, but hourly routine observations of each individual animal on test are implemented, during which if an animal is determined, through the onset of symptoms, to be unlikely to survive until the next set of observations, or the end of the test, they will be removed and killed by a Schedule 1 method to reduce animal suffering. Occasionally animals may be singularly housed for short periods of time towards the end of a study if the animal does not show symptoms or signs of ill health.

Occasionally mis-injection into the lumen of the intestine could potentially cause peritonitis. Occasional injection into the bladder can occur. Careful injection by experienced technicians reduces these risks and any animal suspected of being mis-injected will be killed by a schedule 1 method. All animals except possibly those in the very low dose groups will show typical signs of the product to some degree; this includes difficulty with breathing (deep gasping or abdominal breathing), lethargy, piloerection, an inability to move and some limb paralysis. Some animals will recover from these signs over the course of the test so all need to be kept alive until they are showing severe clinical signs of toxicity at which point they are killed by a schedule 1 method.

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

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

The expected severities on this project are mild, moderate and severe.

It is anticipated that approximately 50% of the animals will experience severe symptoms. We work hard to ensure that animal suffering is as reduced as much as possible, and intervene using a humane-end point opposed to lethality. The remaining animals are likely to experience either a mild severity (mild symptoms following dosing and recovery over duration of test) or moderate severity (symptomatic but able to eat, drink and move, possible recovery over duration of test).

What will happen to animals at the end of this project?

- Killed

A retrospective assessment of these predicted harms will be due by 12 October 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?



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 mode of action of toxins is complex and is a challenge to fully replicate in cell culture or with other in-vitro techniques and the current animal model has been regarded as the 'gold-standard'. Therefore, development of non-animal alternatives has been challenging, especially when comparing to the mouse LD50 assay. Neurotoxins are proteins that have similar molecular structure and molecular weights. They have a di-chain structure consisting of a light chain, which is the toxic portion of the molecule, and the heavy chain which is responsible for targeting the cholinergic neurons. These neurotoxins act presynaptically by blocking the release of the neurotransmitter, acetylcholine, at the neuromuscular junction. Capturing this mode of action in a cell-based/non-animal method proposed challenges and even with the development of non-animal assays there is still currently an ongoing need for the mouse potency assay. This is true for manufacturers who have yet to successfully develop an alternative as a replacement, for high potency products (where alternatives may not be sensitive enough) and as a "back up" to ensure product availability due to non-animal assay failure. There may also be a requirement to use the mouse assay for the qualification of reference standards and the comparability of both in vivo and in vitro assays. Some manufacturers have developed toxin specific cell culture assays, but these methods are not always available to other manufacturers, and it may not be possible to validate other toxins, even those of the same serotype.

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

It is important to address that sponsors of this project licence have cell-based/non-animal methods either approved or awaiting approval and all work carried out under this licence is for product release into territories in which the alternative method has not yet been accepted, or for which an alternative has not yet been developed or approved.

All clinical doses and responses are monitored in terms of the LD50 unit and any attempt to introduce new alternative units would compromise the safety and efficacy of the products. For various reasons relating to the way that this toxin works, these values are considered as a fundamental property of the product which, using a predefined assay system, gives good control of the product.

Alternative methods that are developed need to be able to report an equivalent LD50 result in order that clinicians may continue to use the product in a safe efficacious manner and thus there will still currently remain a requirement to assay a limited number of batches of product by the mouse LD50 method to produce reference standards for use in other assays.



Alternative in-vivo assays have also been investigated; an assay involving hind limb paralysis in mice and proposed a new unit called the median paralysis unit. This does not directly correlate with the LD50 value but is claimed to give a clinically significant determination of the activity. This is not a simple method to use routinely.

Sesardic (Sesardic D, et al, Refinement and Validation of an Alternative Bioassay for Potency testing of Therapeutic Botulinum Type A Toxin, Pharmacology & Toxicology, 1996, 78, 283-288) has reported an assay involving subcutaneous injection of sub lethal doses of the toxin and evaluated the flaccid paralysis caused at the site of injection. Although the assay is reported to give good correlation with the LD50 values, it appears to be less precise and nonlinear.

A Flaccid Paralysis Assay was developed based on the research of Sesardic. The sensitivity was adequate, and some reproducibility was possible, but accuracy was a definite problem. Development of the Hind Limb Assay was considered but progress was being made on cell assays and the message from some quarters (the FDA in particular) was that only a complete animal replacement assay would be accepted rather than one of reduced severity. With this knowledge, the use of further animals to develop non-lethal assays could not be justified.

Currently, efforts are largely focused on cell-based assays as the replacement for the mouse assay. Most major manufacturers have or are currently registering these methods with marketing authorisations.

Sponsors of this licence have worked over several years to develop non-animal methods to replace the mouse LD50 and as marketing authorisations accept the cell-based assay the use of the mouse assay will decrease dramatically. As discussed above for the foreseeable future there is likely to remain a need for the mouse assay.

Why were they not suitable?

Some in vitro alternatives have been approved and been considered suitable and for territories in which the alternative has been accepted, the product is not tested using animals. The animal model is still required until all regulated territories accept alternatives as suitable to ensure the continued safe production of clinical products.

A retrospective assessment of replacement will be due by 12 October 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your experience?

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 currently required per annum is based on the test history of these assays at this facility and current requirements of sponsors of this licence.

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

The animal numbers required for the potency assay has been reduced as experience has been gained at closely targeting the expected values. Careful design of the assays using a geometric progression of dilutions that results in a symmetric design about the known estimated potency ensures a robust assay with maximum precision from the number of mice used in the assay designed to be appropriate to meet the regulatory requirements to safely control the production and release of the product.

The majority of samples received for potency assay are determined by the Routine Quality Control (QC) Assay. This now uses between 200 and 480 mice with no preliminary range finding assay as opposed to 672 mice used in the initial quality control assay.

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

Animals are only received into the facility if testing requirements are confirmed and approved. Technicians carrying out procedures are expertly trained to reduce error and cause for repeat testing and customers are required to provide justification for products before tested.

A retrospective assessment of reduction will be due by 12 October 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

Refinement

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



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

Historically the majority of the work undertaken on biological toxins has been with mice as the animal model considered the lowest neurophysiological model appropriate.

The mouse lethality assay for the biological toxin test requires death as an end point; however, suffering can be reduced by killing, using a Schedule 1 method, any animals that it is predicted will die during the course of the test. Mice are observed at regular and frequent intervals, those showing severe symptoms will be killed. Approximate proportions of animals experiencing mild, moderate and severe severity are 11%, 38% and 51% respectively. However regular observations ensure that approximately 90% of mice experiencing severe symptoms/severity are humanely killed before death from the effects of the toxin.

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

Mice are considered to be the of the lowest neurophysiological sensitivity that will allow us to achieve the projects aims. It is not possible to use terminally anaesthetised animals due to the required assessment of onset symptoms as a result of exposure to the products tested.

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

Hourly observations are implemented to ensure all animals on test are checked to minimise pain and suffering. Each individual is observed and animals showing severe symptoms of toxicity/paralysis are considered unlikely to survive until the next observation period. Animals where these symptoms are clearly observed, and it is considered the animal will not survive to the next observation period, will be killed by a schedule 1 method. Intensive training of staff undertaking observations allows a high degree of consistency between staff members in identification of animals that have reached the end point, particularly in the early part of the test. Further intervention based on clinical signs is not feasible at this stage as not all animals expressing some of these symptoms will die during the stated time point of the assay. Removing animals that would survive the duration would influence the test giving an untrue high result. The assay was originally an LD50 but it has been highly modified over time with the use of early intervention using humane endpoint determination. The requirement for the test however remains a lethality assay and changing for instance to a time to death assay would not gain regulatory acceptance, certainly within the time frames expected for large scale replacement of this assay with in vitro methods.

During the life of the two previous licenses the performance on early humane endpoint determination has been monitored. In 2009 approximately 25% of animals were removed early from the tests, although at this time the humane endpoint was not monitored



routinely. Since then, the early removal of animals has been monitored using a reference standard assay as this is of known potency and performance and should not vary greatly week to week. The reference assay is only performed alongside testing of Drug Product, using 240 mice. This is not an additional assay to assess humane end-point, the data is collated from assays required to test Drug Product. Our humane endpoint success rate is on average 91% (range of 84% – 94%). This means that in a standard assay where half the animals will not be alive at the end of the assay, an average of 109 will be humanely killed whilst 11 will die in extremis. Performance of individual staff members is reviewed to ensure they are maintaining the standards required and are successful in removing animals at an early enough stage. Because the survival of an individual mouse will change the potency, it is important to ensure staff do not over cull animals as this could bias the results and lead to repeat assays being required. A good understanding of relevant symptoms is critical to the success of this procedure.

The assay has also been refined in that wet mash is supplied to animals. This allows for easier access to food and water and provides a more palatable system for animals that are affected by the product. In the past year, nesting material has been successfully introduced into testing cages, to allow the mice housed in test cages to demonstrate typical nesting behaviours.

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

We perform in vivo testing for clients using methods which are performed in compliance with relevant EU, UK and US regulations and guidelines. Compliance to these standards allows our clients to apply for licencing of their products with a wide range of national authorities including EMEA (European Medicines Agency), MHRA (The Medicines and Healthcare Products Regulatory Agency) and the FDA (US Food and Drug Administration).

All testing is completed to GMP (Good Manufacturing Practice) standards, European Pharmacopeia and ICH guidelines.

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

This will be achieved by regular discussions with our Name Information Officer and by attending appropriate training courses and conferences. I work closely with the establishment NAWCO, AWERB to ensure I stay up to date with advances which may provide refinement in this project which will be applied where possible.

A retrospective assessment of refinement will be due by 12 October 2029

The PPL holder will be required to disclose:



- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?



5. Studies of Leukaemia Immunology and Stem Cell Transplant Biology

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

Leukaemia, Transplantation, Immunology, Immunotherapy

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

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 immune system eliminates leukaemia after successful stem cell transplantation (a treatment in which diseased bone marrow cells are replaced with healthy cells from a donor), why this sometimes fails and what can be done to increase the chances of success.

A retrospective assessment of these aims will be due by 25 October 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence? Did the project achieve its aims and if not, why not?



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 recent revolution in immunotherapy (treatment through manipulating the patient's own immune cells) for cancer has yet to improve the outcomes of patients with myeloid malignancies (blood cancers), whose outcome remains poor. Stem cell transplantation is the only curative therapy for many patients with acute myeloid leukaemia (AML) and other cancers of the blood and bone marrow.

However, cancer recurrence remains the most common cause of death, and is due to failure of the donor immune system to eliminate residual disease. The immune cells most responsible for clearing leukaemia are T cells. T cells are often dysfunctional at relapse, and leukaemia is frequently able to evade them. This project aims to understand why T cells become dysfunctional, and how AML escapes them. This is critical to the development of new treatments that re-establish immune responses to treat or prevent relapse. Leukaemia is a cancer of cells that would normally become mature blood cells through a process called differentiation. Some of these mature cells are capable of stimulating T cells and triggering immune responses. Several new drugs can induce leukaemic differentiation, and we wish to establish whether they can be used to create beneficial anti-tumour responses.

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

Expected outputs of this project include new knowledge regarding the mechanisms used by leukaemia to escape donor immune cells following stem cell transplantation and novel strategies for improving anti-tumour immune responses. This knowledge will be shared through peer-reviewed publication and presentation at scientific meetings. This work is directly translational and is intended to inform the design of clinical trials.

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

- **Patient beneficiaries:** The primary aim of this project is to benefit patients with myeloid malignancies undergoing stem cell transplantation. These patients face a significant risk of treatment failure and death. The most direct route to impacting patient care is to use compounds that induce leukaemic differentiation and are already approved by regulatory authorities, then reposition them for use in the post-transplant setting (assuming that data arising from this project supports the hypothesis that inducing differentiation can augment donor immune responses). However, the intention of this project is to generate the pre-clinical evidence to support a future trial and it is unlikely that any clinical trials would be performed during the 5 year duration of this project.
- **Scientific beneficiaries:** Understanding how leukaemic cells evade donor immune responses is of primary importance to the leukaemia research community. Data arising from this project will also be of significant interest to cancer immunology researchers more broadly, as defining mechanisms of T-cell dysfunction is central to understanding the action and limitations of contemporary cancer immunotherapies. Scientists will benefit from the dissemination of this new knowledge through publication/presentation and also through the sharing of datasets via established repositories.



- **Clinical beneficiaries:** Findings will be of direct relevance to the clinical transplant community and will inform the design of clinical trials that attempt to improve donor T-cell responses after transplant. Again, the intention of this project is to generate the pre-clinical evidence to support a future trial and it is unlikely that any clinical trials would be performed during the 5 year duration of this project.
- **Public beneficiaries:** Transplantation is an expensive treatment (~£230m per year in England) and a significant proportion of this cost arises from managing complications and treatment failure. Increasing the effectiveness of this therapy therefore represents a potential cost saving to the health service.
- **5. Industrial beneficiaries:** The repositioning of existing compounds will be of interest to several pharmaceutical companies, broadening the potential market for their drugs and attracting further investment in this area.

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

Findings will be made available to other scientists through collaboration, peer-reviewed publication and presentation at scientific meetings. Where applicable, datasets will be shared with other researchers via established repositories, for example the Gene Expression Omnibus (GEO) which collects DNA and RNA sequencing data to make available to other researchers. Our Establishment has a policy of ensuring that all publications are open access.

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.

The development and function of an immune response involves many different cell types interacting in a dynamic three-dimensional environment. For example, the progression of an immune response within a whole body involves changes of antigen (a molecule that can trigger immune responses) expression and presentation that evolve with both time and spatial distribution. Similarly, cancer development and spread involves a plethora of interactions between cancer cells and their surrounding cells, governed by multiple chemical signals originating from both their immediate neighbours and from distant tissues. These factors, combined with the involvement of multiple host cell-types and the expansion and migration of rare tumour-specific immune cells, mean such research cannot be carried out in tissue culture alone or reproduced using computer modelling (in silico) and can only be addressed with the use of animals.

The mouse is one of the model organisms that most closely resemble humans. The human and mouse genomes are approximately the same size, and display an equivalent number of genes, which are functionally conserved. Further, mice have genes not represented in other animal model organisms (e.g. *Caenorhabditis elegans*, i.e. nematode



worm, and *Drosophila melanogaster* i.e. fruit fly) such as those involved in adaptive immune responses (the processes that provide memory of specific antigens). Mice can be genetically altered, there is extensive literature concerning the topics of our investigation, and our own studies can be enhanced by combination with many complementary models developed by others in the field. Definitively, mouse models are important for placing the findings of in vitro (test tube) studies or analysis of human samples into an appropriate and meaningful context, that of a living organism. It is the combination of in vitro, in silico and in vivo studies that provides the insight needed to understand cancer biology and develop new therapeutic approaches, and there are no effective approaches to hand that can replace the in vivo studies, as only these allow the in vitro findings to be tested in an environment that contains all of the elements necessary to determine the outcome of an immune response. For our studies we need animals with a functionally mature immune system, therefore we will only use adult mice.

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

A typical experiment would involve brief irradiation of mice with X-rays followed by injection of blood cancer cells into the tail vein or under the skin (subcutaneously). Some experiments would involve treatment of mice with drugs by mouth (typically daily for 2-3 weeks) or by injection. Leukaemia development will be assessed either by blood-cell count monitoring every four weeks or, in the case of subcutaneous tumours, using callipers to measure them. Some mice will have specific populations of immune cells injected into the tail vein. Typically, a single mouse will not experience more than 4 types of procedure. The health of all mice will be observed daily. All mice will be killed humanely at the end point of the experiments.

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

The proposed procedures (X-ray treatment, anaesthesia, injections and blood sampling) are mild in severity and are associated with, at worst, only slight or transitory minor pain or suffering. Occasionally (5-10% of procedures) after full dose irradiation some mice might lose weight for several days before regaining it, and so these procedures are considered moderate in severity. Additionally, some mice will experience the discomfort of repeated (daily) injections or oral delivery of therapeutic agents, typically for 2-3 weeks, this is considered moderate in severity. We will aim to utilise the least stressful route of administration wherever possible. The health of all mice will be observed daily. Mice injected with blood cancer cells will, when the disease develops, exhibit signs of disease. Mice will be culled when they exhibit signs of advanced disease, such as hunched posture, poor levels of socialising and interaction. Under these circumstances, and whenever else a mouse displays features of ill health, or at the end of each experiment, mice will be killed humanely using a Home Office authorised method. Despite close monitoring, a small proportion of mice (2-5%) with leukaemia are found dead in their cages, sudden death is regarded as a severe adverse effect.

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 leukaemia models described here are overseen by a team of very experienced licensees. Based on our collective experience of using these procedures and experimental models we anticipate that 85- 90% of mice will experience mild severity, 5-10% moderate and 2-5% severe. Thus, the majority of mice are only expected to experience mild symptoms related to tumour growth before they are humanely killed.

What will happen to animals at the end of this project?

- Killed

A retrospective assessment of these predicted harms will be due by 25 October 2029

The PPL holder will be required to disclose

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

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 development and function of an immune response involves many different cell types interacting in a dynamic three-dimensional environment. For example, the progression of an immune response within a whole organism involves changes of antigen expression and presentation that evolve with both time and spatial distribution. Similarly, cancer development and spread involves a plethora of interactions between cancer cells and their surrounding cells, governed by multiple chemical signals originating from both their immediate neighbours and from distant tissues. These factors, combined with the involvement of multiple host cell-types and the expansion and movement of immune cells around the body, mean such research cannot be carried out in tissue culture alone or reproduced in silico and can only be addressed with the use of animals.

The mouse is one of the model organisms that most closely resemble humans. The human and mouse genomes are approximately the same size, and display an equivalent number of genes, which are functionally conserved. Definitively, mouse models are important for placing the findings of in vitro studies or correlative analysis of human samples into an appropriate and meaningful in vivo context. It is the combination of in vitro and in vivo studies that provides the insight needed to understand cancer biology and develop new therapeutic approaches, and there are no effective approaches to hand that can replace the in vivo studies, as these allow the in vitro findings to be tested in an appropriate environment.

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

Non-animal alternatives include cancer cell lines, primary patient material and healthy donor immune cells. We routinely use these in our research to identify compounds that induce differentiation in leukaemic cell lines and patients samples. We then study the interactions of these leukaemia cells with different immune cells to identify treatments that



result in enhanced immune cell activation. As well as identifying the most promising treatments, these experiments inform the design of animal experiments by showing us which immune cells we should study and how much time is required for the desired effects to be seen. This reduces the number of mice needed for pilot experiments or protocol optimisation.

However, the overarching aims of this project cannot be met without using living organisms. There are no suitable alternatives to address our research questions and achieve our goal or translating our research into treatments for patients, because organoids or microfluidic platforms that simulate some of the cellular interactions within certain tissues cannot recapitulate the outcome of an immune response in a whole organism.

Why were they not suitable?

Only a mammalian organism has the potential to accurately mimic human normal/leukaemic blood cell production and adaptive immune responses. We use all of the methods described above to formulate and investigate hypotheses, but none can recapitulate the complex and dynamic interactions between multiple cell types across several tissues that are necessary to investigate the relationship between leukaemic cell states and the immune populations that sustain or eliminate the disease.

A retrospective assessment of replacement will be due by 25 October 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your experience?

Reduction

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

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

The overall aim will be to generate models whereby a measurable effect e.g. reduction in blood cancer cell burden or incidence following manipulation of a gene of interest or treatment with a drug or immune cell treatment can be determined using the minimal number of animals.

Based on past experience, group sizes of between 5 and 10 animals (depending on the investigation) per experimental group suffice. For instance, in experiments where we deplete a gene in a cell type by genetic manipulation in the test tube, we might use two independent approaches targeting the gene as well as a control for that approach, and potentially several doses of a drug, or several different drugs or treatment combinations to test a hypothesis. We have estimated the total number of mice to be used over the licence lifetime, taking into account the previous experience of other licence holders in the establishment working in the same scientific area.



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

- The use of mice will be minimised in several ways:
- By using statistical methods to calculate the minimum number of animals needed to confirm or refute our hypotheses.
- By incorporating as many test groups as possible within a single controlled experiment, reducing the number of controls required compared to a series of smaller experiments.
- By utilising tissues and tumours from different sites on one mouse for both treatment and control samples.

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

The use of mice will be optimised in several ways:

- Breeding is performed on a different project licence by a dedicated group in our Establishment. We will work closely with this team, informing them of our plans for the coming months so that they can help to maintain colonies of an appropriate size and avoid the breeding of more mice than we require.
- By doing as much preliminary work as possible in culture models in vitro and in silico analysis prior to engaging in in vivo studies.
- By minimising variability in results through utilising inbred strains, to generate as near as possible “identical” mice and by housing them under similar conditions.
- By performing pilot studies using few mice when no information is available in the literature so that the number of mice utilised in experiments is reduced to minimal levels.
- In all new experimental models and protocols, we will establish the base line by procuring help and advice from animal care and veterinary staff and researchers at the establishment, but also from our experienced outside collaborators across the UK and internationally. Furthermore, we will design small pilot experiments, carried out referring to the principles of <https://www.nc3rs.org.uk/conducting-pilot-study>, that will allow us, where appropriate, to select the ideal cell type so fewer animals are used, to calculate the minimum number of animals required for an experiment, given the rate of expected events, and also to determine the severity of these events, allowing more accuracy for statistical powering calculation of group sizes in potential repeats. Lastly, pilot experiments, potentially for all aspects of the Protocols, will enable us to better plan the duration and size of the experiment and to help monitor for side effects at the critical time points.

A retrospective assessment of reduction will be due by 25 October 2029

The PPL holder will be required to disclose:



- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

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 common models of T-cell immunity, such as the OT-I and OT-II mice (these have T cells that recognise a specific protein that cancer cells can be engineered to express). The use of very well characterised models will reduce the number of pilot and optimisation experiments required before usable data can be generated. The use of inbred mice reduces intragroup experimental variability, permitting smaller numbers of mice to be used in each experiment and also eliminates incompatibility when cell transfers are performed.

When the first mice develop leukaemia (the 'primary' transplant), we will harvest leukaemia cells and use them to initiate leukaemia in subsequent mice ('secondary' transplants). The advantage of this approach is that the secondary transplants develop leukaemia much more quickly (~20 versus 100 days), which reduces the amount of time mice spend being monitored and sampled. The development of leukaemia is also more predictable, allowing interventions to be planned on specific days, further reducing the amount of monitoring and the chances of mice being wasted by performing interventions either too late or too early in the development of the disease.

Mice occupy an enriched environment and are cared for by staff trained and expert in animal monitoring and handling. Mice will not be handled by the tail. For experimental approaches involving drug treatment, the least invasive route is used where possible (e.g. an oral route rather than intravenous (into a vein) or intraperitoneal (into the abdomen) where a drug has high oral bioavailability and is stable in water); mice which undergo anaesthesia and bone marrow puncture will be treated with fluid and analgesia to minimise risk of pain and distress.

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

Other less sentient non-mammalian species, such as zebrafish or frog, which lack a haematopoietic system that is comparable in complexity and anatomy to that of humans have been considered and rejected as models. There is also no published experience to date on the transplantation of human haematopoietic cells into fish or amphibians. Only a mammalian blood system has the potential to accurately mimic both the anatomy and complex cell biology, including local cell-cell and cell-chemical (microenvironmental) interactions, of human normal and blood cancer cell tissue. One of the drawbacks of experiments using human blood cancer cells is their genetic variation makes the accurate and meaningful study of the effects of specific genetic lesions in isolation very difficult. By contrast, murine models of human leukaemia enable investigation of the biological effects



of specific genetic lesions in a tractable, controlled and highly informative manner. Adults need to be used because they have a fully developed immune system whereas mice at an earlier stage of development do not. Multiple mouse models exist to provide antigen specific and tractable immune populations as well as models lacking specific immune populations. These models permit the dissection of complex immune phenomena, and are not available in other species.

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

The techniques used have been carefully evaluated and refined by others at the Establishment to minimise distress to the animals. Mice used in surgical procedures will be treated with anaesthesia, analgesia and post-operative rehydration by subcutaneous injection, followed by careful observation. In other areas, irradiation doses will be administered at a level sufficient to induce bone marrow suppression but no other long term impact; higher doses of X-ray irradiation are delivered in split doses; bone marrow injections and sampling will not be performed routinely, only where the scientific justification is high; and in studies that result in the initiation of blood cancer, mice will be closely monitored for health status and killed by a Home Office approved method when signs of ill health are displayed. When considering which route of administration of substances to employ, we will strive to use the least invasive route whilst maintaining direct control of dose. The choice of route to administer a drug or cells will be such as to achieve "best practice", i.e. to minimise or avoid adverse effects, reduce the number of animals used, and maximise the quality and applicability of substances and cells to achieve the scientific objectives.

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

Surgical procedures will be carried according to the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery. Unless otherwise specified, this project will follow the "Guidelines for the welfare and use of animals in cancer research" and the administration of substances and withdrawal of blood will be undertaken using a combination of volumes, routes and frequencies that of themselves will result in no more than transient discomfort and no lasting harm (Morton et al., Lab Animals, 35(1): 1-41 (2001); Workman P, et al. British Journal of Cancer, 102:1555-77 (2010)).

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 local 3Rs events and through discussing refinements with our animal care staff and vet. I will also read the NIO reports that are circulated within the Establishment. I will also attend and contribute to our Retrospective Review and Licensees meetings and the 3Rs Poster session, all of which take place annually at our Establishment.

A retrospective assessment of refinement will be due by 25 October 2029

The PPL holder will be required to disclose:

With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?



6. Inter-organ communication and glucose homeostasis

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

Diabetes, Cytokines, Metabolic tissues, Insulin, Glucose homeostasis

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

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

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 main aim is to comprehend the cellular and physiological mechanisms that drive the development of diabetes so we can improve the treatment of this disease.

A retrospective assessment of these aims will be due by 1 November 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?



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?

More than 4.9 million people in the UK has diabetes and this number is predicted to increase to 5.5 million by 2030. Around 90% of people with diabetes have type 2 diabetes and around 8% have type 1 diabetes. Type 2 Diabetes (T2D) and its complications contribute to poor quality of life, increase vulnerability to infections like COVID-19 and premature death. The National Diabetes Audit 2021-2022 quarterly report for England reported that only 35% of patients meet the treatment targets for Hba1c (the average blood glucose levels for the last two to three months). There is therefore an unmet need to develop a more effective approach to understand and tackle the pathophysiology of T2D. The specific biological factors that contribute to diabetes are not well comprehended. The pancreas contains beta cells, which release insulin in response to rising glucose levels after a meal. These cells, along with alpha cells that secrete glucagon to counteract insulin's effects during low glucose conditions, are found in the islets of Langerhans within the pancreas. It is widely acknowledged that inadequate insulin secretion by beta cells plays a role in the development of diabetes. Additionally, various metabolic tissues, such as the liver, fat, muscle, and brain, collaborate to maintain appropriate glucose levels throughout the body. Diabetes is characterized by abnormally high blood glucose levels (hyperglycemia), which can lead to life-threatening complications such as ketoacidosis (potentially resulting in a diabetic coma), severe dehydration, and long-term damage to the eyes, kidneys, nerves, and blood vessels, among other effects. A better understanding of the mechanisms underlying diabetes and the effectiveness of current treatments is crucial for the advancement of new and improved therapies for this condition. One prime example that will be explored in this Project License is bariatric surgery. Bariatric surgery is the only treatment that can cause total remission of T2D, compared to medical treatment. The first randomised controlled trial demonstrated 37.5% maintained diabetes remission throughout 10 years post-surgery, compared to 5.5% in patients on medical treatment (Mingrone et al, Lancet, 2021). Importantly, this effect does not seem to be strictly due to weight loss, as studies comparing patients post surgery and patients on very low calorie diet demonstrate greater effects on blood glucose in the surgical group (Salem et al, Diabetes Care, 2021). Unraveling the mechanisms behind this effect can allow us to develop less invasive treatment options such as medical treatments with similar potency, not only for obesity and diabetes, but also for other associated metabolic diseases.

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

This project will provide new knowledge into the mechanisms that control how the different organs in the body work together to maintain an adequate level of sugar in the blood and why they fail to do so in diabetes.

Specifically, the main specific outputs of this project are:

Identification of new molecules ("made from specific pieces of the DNA, or "genes") that are important for the control of glucose levels and the development of diabetes

Understanding why those genes are important and whether they are involved in the efficacy of certain diabetes treatments.

Better understanding of how specific treatments can work for diabetes, as multiple diabetes drugs are approved for treatment, yet their exact and full mechanisms of actions remain



unclear. This includes some commonly used drugs (i.e. exendin-4, SGLT2-inhibitors).

Investigate effects of bariatric surgery in glycemic control and metabolic homeostasis, beyond weight loss, by focusing on multiple organs rather than the pancreas only.

Publications and conference presentations that will provide wide-access to this new knowledge

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

The whole scientific community will benefit following the publication of our studies and the presentation of our data in scientific meetings (as early as 6 months after the start of the studies). The genetically modified animals generated will be made available to other researchers. Genetically modified animals will be bearing a small piece of DNA generated in the lab which will make them have more or less amount of the protein (or other biological molecules such as RNA) of interest. In this projects these molecules of interest will be those suspected to be important for the development of diabetes. The techniques used here will also be shared with the scientific community. This is important since, for example, one of the protocols described within this project provides the ability to monitor the development of diabetes and the function of the pancreatic islets over time in a non-invasive manner, in the living mouse. This means that the same animal can be studied at different time points with minimal disturbance and allows the reduction of the number of laboratory animals used, since regular killing at serial time points to assess the same parameters is not required.

The research proposed here is basic, or also called fundamental research, with the aim of improving scientific theories for better understanding on why diabetes occurs and how we can treat it so we don't anticipate a benefit to the pharmaceutical industry in the short term. Nevertheless a better understanding of the mechanisms contributing to the development of diabetes, as well as the benefits of bariatric-surgery to improve this disease will, in the medium-longer term, favour the generation of better, more refined treatments for diabetes and other metabolic diseases. This will therefore benefit the pharmaceutical industry and the patients suffering these diseases. The American Diabetes Association guidelines accept that bariatric surgery causes diabetes re-emission in 80% of cases but this is not without significant changes in the patients style of life. Our research may contribute to create more specific drug targets that could allow us to replicate the effect of bariatric surgery in a less invasive way (e.g. Injection of gut-derived peptides). We will also be working closely with human bariatric surgeons and endocrinologists to see how our findings can contribute to optimise patient surgical and medical treatment in Type 2 Diabetes.

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

As mentioned above, we will disseminate our data by publishing it in peer-reviewed journals. We will also generate and publish reviews and in-detail methods that will allow others to use the techniques optimized by us under this project licence. This will allow us to also showcase our means for troubleshooting and unsuccessful approaches. We will also share our research and new knowledge in local and international meetings and we will be available to train members of our own teams, and others within our institution. This will boost the establishment of new collaborations.

When possible, we will store tissues obtained with these studies to distribute to different



researchers to ensure maximum effect and animal reduction. For example, in experiments performed with bariatric surgery, the kidneys can be used for glucose transport research, stomach for bile acid research, liver for hepatic insulin resistance research, etc.

Species and numbers of animals expected to be used

- Mice: 7000
- 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.

Mice are the lowest vertebrates in which genetic manipulation can be successfully achieved and where diabetes studies are well documented. Rats give a better yield of blood and tissues per animal than mice and could be preferred if the relevant strain is available.

Both species are well acclimated to live in cages and laboratory conditions.

Adult animals are used in all experimental protocols since full control of glucose levels in the body is observed in adult animals. All life stages (adult, embryo, neonate, juvenile, pregnant) are required to generate genetically modified mice in order to fulfil the objectives of this project in the breeding and maintenance protocols.

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

Most animals in our project will be genetically modified. In some cases, the genetic modification will alone promote the development of mild diabetes, defined as non-fasting glucose levels in blood $> 13\text{mM}$ and characterized by excess urination and weight loss but, often, we will need to promote the development of the disease (to see whether our gene of interest delays or worsens diabetes). To do so we will administer "diabetogenic substances" which are chemicals that promote the destruction of the cells that produce insulin (beta cells) and therefore produce defects similar to diabetes type 1 (T1D).

This will be done if we suspect a specific gene is involved in the progression of beta cell apoptosis or regeneration, which will be highly beneficial in beta cell physiology investigation. More often, we will feed the animals a high fat, high sugar diet. This diet mimics a westernized diet and promotes obesity and type 2 diabetes. The animals will be monitored for the development of diabetes by using standardized methods, including glucose tolerances tests and blood sampling, similar to what occurs to humans in the clinic.

Finally, a small percentage of our animals (~3%) will undergo bariatric surgery, a procedure that is performed in the clinic to humans to treat obesity and diabetes. Bariatric surgery consist in surgical removal of part of the stomach so the animals don't eat as much and lose weight. Due to weight loss and/or other mechanisms that are still unknown, diabetes remits. Animals will undergo this surgery and then will be evaluated for the development of diabetes by using the standardized methods mentioned above.



At the end of the studies, animals will be humanely killed and the tissues will be extracted post-mortem for further studies.

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

The vast majority of our animals will undergo very low adverse effects (defined as "mild") during their breeding and maintenance.

Animals will experience temporary discomfort related to the specific procedures performed (for example, overnight fasting, followed by an injection and a small cut or needle prick to extract a drop of blood during glucose and insulin tolerance tests) or to the development of diabetes. Development of diabetes may include chemical ablation of pancreatic beta cells, or exposure to high fat diet. The clinical symptoms associated with diabetes that we might observe in these rodents are minor weight loss or weight gain, and excessive urination. Osmotic pump implantation may also be performed in some animals, and a brief post-operative period of close monitoring is expected. These procedures account for a higher level of severity and are defined as "Moderate".

Animals undergoing bariatric surgery (~3%) may present more severe adverse effects with a mortality rate of up to 30% by humane killing due to the adverse effects including bleeding inside the abdomen or stomach, intestinal obstruction or leaks and small hernias. The symptoms for this include dark faeces, hunched posture, erection of the hair of the skin ("piloerection"), social isolation, failure to groom, failure to feed/ drink, dark pigment discharger under eyes and nose and enlarged abdomen. In order to avoid such adverse effects, surgery will be performed aseptically by trained personnel, with close support from the NVS and the NACWO.

Appropriate anaesthesia and subsequent pain relief, antibacterial bedding, and liquid diet will be supplied. Animals will be monitored several times a day and animals demonstrating severe symptoms will be killed to prevent further 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)?

Mouse: Mild: 64.6% Moderate: 35% Severe:0.4% Rats: Moderate: 75% Severe:25%
What will happen to animals at the end of this project?

- Killed

A retrospective assessment of these predicted harms will be due by 1 November 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

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 maintenance of normal blood glucose levels requires the coordinated action of several metabolic tissues (i.e. liver, fat, pancreas, etc) that secrete and respond to hormones performing actions that regulate the use of glucose by the body. Also the gut and the brain play very important roles in sending signals in response to food availability to control the production and response to hormones of the other tissues.

These complex interrelations cannot be precisely reproduced in a petri dish (in vitro) and require a whole living organism.

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. Cell lines will be used whenever possible, to reduce the number of animals used in this project. For example, cell lines will be used to obtain preliminary data that help us identify genes that are likely to be involved in the development of diabetes.

We obtain human samples from patients undergoing bariatric surgeries that can be used for some of our studies.

We also use computational programmes to predict gene function and to simulate scenarios and analyse data whenever possible.

In the future, we will attempt to utilise organ-on-a-chip technology,. However, this would only allow us to interrogate one organ rather than the multi-organ interaction, which is an overall aim in the license.

These approaches will always be used to replace experiments on animals if at all possible.

Why were they not suitable?

It is not currently possible to mimic the interplay between all metabolic organs, including the pancreatic islets and the brain, for the study of metabolism and diabetes. For example, it has been demonstrated that the hormonal changes that occur as a result of partial gut removal during bariatric surgery don't act directly in all other tissues, but can have several indirect effects thought several tissues: for example gut hormones affecting liver function that affects pancreas function to control insulin secretion. It is therefore impossible to fully facilitate cell lines and isolated tissues to replicate the effect of bariatric surgery in vitro without an initial in vivo assessment. Also, our research will require extensive access to post-operational intestinal tissue in specific days during the study, which is not feasible with human subjects who undergo bariatric surgery.

A retrospective assessment of replacement will be due by 1 November 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started,



and is there anything others can learn from your experience?

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 statistics to predict how many animals will be needed in each of the different protocols so we can fulfill our objectives in the five year period. The data used to perform this statistical analysis has been obtained from similar experiments performed by us in the past or from similar published work done by others.

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

We have performed power calculations, which are mathematical calculations that predict how many samples (and in this case, how many animals) need to be used in a specific type of experiment so that when comparing different treatments or conditions, we are likely to detect an effect that is not due to chance.

Power calculations have been done with specific software such as Gpower and with other available tools (such as the NC3R's experimental Design assistant) to determine the minimal number of animals required to obtain reliable results, and to ensure that no more animals than the strictly required are used in experiments.

These tools will be used by all researchers working under this licence throughout the duration of the project to reduce the use of animals as much as 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 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 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, to perform experiments in the laboratory so no additional animals are required.

A retrospective assessment of reduction will be due by 1 November 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

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 mice and, occasionally, rats (see justification below)

Most of our mouse models will be genetically modified so we can study the function that specific genes play in the development of diabetes and its treatment. We will also use diabetes models. Type 1 diabetes is characterized by the loss of the cells that secrete insulin (pancreatic beta cells within the islets of Langerhans) while type 2 diabetes, which comprises 90% of the cases, involves malfunctioning of these cells and the lack of response to insulin of other metabolic organs. We will use models to mimic these types of diabetes (more often, T2D). For T1D we will inject substances that produced a controlled degree of beta cell death and, for T2D, we will feed our mice a "westernized" diet, consisting of high sugar and/or high fat content, which is well tolerated but leading to obesity and type 2 diabetes over time. We use these models because they mimic very well the human disease and they are easy to control, infringing the minimal suffering in the animals.

We will use several experimental methods to monitor diabetes. The most commonly used method will be blood sampling to measure glucose and hormones (such as insulin). Very often, we will perform glucose tolerance tests where we will administer a bolus of glucose to the animals and we will measure, via sampling from their tail vein, the concentration of glucose in their blood at different times after. This will tell us whether the animals are capable of control the levels of glucose in their blood, which they will not if they are diabetic. Additional methods will aim to understand how the genes that we have manipulated, or the treatments or diabetes-inducing substances that we have given work in more detail.

All the procedures in this licence except the bariatric surgery are classified as either mild or moderate, meaning that the vast majority of our animals will undergo very low adverse effects (defined as "mild") in breeding and maintenance, or mostly temporary discomfort related to the specific procedures performed (for example, tail vein sampling to extract a drop of blood) or to the development of diabetes (defined as 'moderate'). The clinical symptoms associated with diabetes that we might observed in these rodents are minor weight loss and excessive urination. Also, procedures are done under local, general or terminal anaesthesia where appropriate to minimise stress and suffering of the animals.

Bariatric surgery is classified as severe due to the high mortality rate (up to 30%) even in trained hands. It is important to note that the vast majority of these animals are proactively killed in order to limit severe suffering, or suffering that cannot be ameliorated. This group of surgeries has been refined by the applicant over the course of 8 years, and include liquid diet, vicryl absorbable sutures, specific sets of high-precision tools, antibacterial bedding, two types of analgesics, injectable and drinking water antibiotics and infrared heating pad. This procedure will only be performed in a small proportion of animals.



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

We will use mice and, occasionally, rats. This is because mice are the lowest vertebrates in which genetic manipulation can be successfully achieved and where diabetes studies are well documented. Rats give a better yield of blood and tissues per animal than mice and could be preferred if the relevant strain is available. We will use adult animals in the experimental protocols since at earlier stages the control of blood glucose is not full and can't fully mimic that of humans and the development of diabetes.

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 relevant data as possible with minimum amount of suffering. We will make all efforts to make animals comfortable whether or not they are undergoing an experimental procedure. This includes giving them a comfortable environment to live and handling them adequately so we don't cause them stress. Animals will also be handled often between experiments to reduce their levels of stress during experiments.

Bariatric surgery has been refined so we use optimal surgery materials (i.e. sutures, tools) and only highly trained scientists will be performing this technique. 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.

For example, animals undergoing bariatric surgery will be monitored, post anaesthesia recovery, every 2 hours during the day and every 4 hours overnight in the first 72 hours as a minimum (more frequently if necessary) and daily thereafter.

Advice will be sought from NAWCO (who is the Named Animal Care & Welfare Officer responsible for overseeing the day-to-day welfare of the animals at our institution), technicians and vets for keeping up to date with best practises and we will use all available guidance (see below) to continue the refinement of our approaches whenever possible.

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

We will follow the LASA (Laboratory animal science association) Aseptic surgical technique and the NC3R's (National Centre for the Replacement Refinement and Reduction of Animals Research) published guidelines including PREPARE (Planning Research and Experimental Procedures on Animals) and ARRIVE (Animal Research: Reporting of In Vivo Experiments), the "Responsibility in the use of animals in bioscience research", "Colony management best practice", "Minimizing the use of GA mice" and the topic-specific resources such as "Anaesthesia", "Handling and restrain".

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

We will be in continuous communication with the veterinary staff in our facility that will, in the case of Bariatric surgery, overlook our studies, and we will provide best advice for keeping up to date with best practises.

As project licence (PPL) holder, I will interact with all named persons and animal



technicians at our institution to review current approaches and whether there are any new 3Rs opportunities. I will continue subscription to the NC3Rs (National Centre for the Replacement Refinement and Reduction of Animals Research) e-newsletter. Myself and/or the other researchers working under this licence will attend selected NC3Rs courses/workshops.

A retrospective assessment of refinement will be due by 1 November 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?



7. Understanding the interactions between host and microbes leading to colonisation, drug resistance and systemic infection

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Antibiotic, Candida, Sepsis, Drug resistance, Immunity

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

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

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 pathways regulating microbial colonisation/infection and subsequent development of antimicrobial drug resistance in vulnerable hosts.

A retrospective assessment of these aims will be due by 1 November 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?



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?

Common life-threatening infections and sepsis often originate from within our own bodies. Microbes living in our guts are normally harmless, but can become dangerous during long-term treatment with antibiotics or other antimicrobial drugs, which is common in patients requiring long-term hospitalisation. Antimicrobials unbalance the microbiome which leads to downstream effects on the immune system.

Moreover, these microbes may acquire new phenotypes that render them resistant to the antibiotics or antifungal drugs within our guts, but how this happens is poorly understood. It's therefore important to understand what influences the growth of these potential pathogens in our guts, and how they change while growing here, to help identify patients most at risk from getting an infection and the likelihood of these infections becoming drug-resistant.

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

The main outputs from this research will be new information on how we might prevent life-threatening systemic infections originating from the gut. We will publish our findings in medical journals as well as magazine articles. We will also present our research at science festivals, public talks and at conferences.

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

The results of our work will be most useful for other scientists in the field, at least in the short-term. For example, our research will lead to the identification of fungal and bacterial strains that allow for modification or tracking of drug resistance in the host, which other researchers may utilise for their own questions in the fields of antimicrobial resistance and gut microbiome.

In the long-term, the findings may be used to inform new treatment strategies and clinical trials that aim to improve how we care for antibiotic-treated patients and the development of antimicrobial drug resistance. Even in the medium term, there is the potential we will generate findings that could influence policy on antibiotic stewardship in hospitals.

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

All of our research findings will be published in journals, including unsuccessful approaches. Some of work includes the use of new microbial strains that allow us to track the development of drug resistance within the gut and understand the external factors (e.g. exposure to antibiotics) controlling this. These new strains will be made available to other researchers upon request, as will raw data so that our efforts need not be duplicated by others unnecessarily. We will also engage the public with our research findings via public presentations (e.g. at festivals) and publishing articles in magazines and popular news sites.

Species and numbers of animals expected to be used



- Mice: 1700

Predicted harms

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

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

We are interested in how changes to the gut that occur with antibiotic treatment affects control of infections by microbes that normally live in the gut as part of the microbiome. Antibiotics can change the microbiome leading to overgrowth of these potentially dangerous microbes (such as pathogenic fungi), but they can also directly impair our immune cells making control of an infection much harder, and poor outcomes more likely. As we are looking at the intersection between drug treatment, the microbiome and immune system, we require a complex mammalian system to answer our research questions since we cannot model a microbiome and complex immune system in vitro. Mathematical models are also currently insufficient to understand the many different impacts that antibiotic treatment has on the body, both at the level of the microbiome and on the immune system. We will therefore use adult mice for our studies, since they have a mature immune system that closely resembles human, and a complex gut microbiome, which is not found in younger or neonate mice. Lastly, mice are a useful model for our work because antibiotics promote the development of antimicrobial drug resistance and immune system defects in these animals, similar to what we have observed in human studies.

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

Mice will be given antibiotics or anti-fungal drugs in their drinking water (or by oral gavage) to model the types of treatments that hospital patients are exposed to, compared to control mice that receive no treatment. We will then provide mice with potential pathogens (bacteria *E. coli* or *Klebsiella*, or the fungus *Candida albicans*) that we have grown in the lab and have a fluorescent tag, so we can monitor how these pathogens grow, develop drug resistance and/or interact with the immune system in the presence or absence of drugs. Pathogens will either be given in the drinking water or oral gavage to model colonisation, or by a single intravenous injection to model systemic infection. In some experiments, mice may also be injected with drugs that suppress their immune system so that we may model the vulnerable status of hospital patients. These drugs mimic the same immunosuppressive regimes used in patients receiving stem cell transplants or cancer therapy, groups who are at high risk of developing systemic infections with drug-resistant pathogens.

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

Mice exposed to antibiotics are not expected to experience any adverse effects, beyond some mild weight loss in the first few days of treatment as they get used to the taste of their new drinking water. In experiments where mice are colonised with bacteria *E. coli*/*Klebsiella* or fungus *C. albicans* (i.e. where pathogens are delivered to the gut via the water or gavage), mice are not expected to experience any adverse effects as these microbes are part of normal microbiota and do not cause illness when their growth is



limited to the gut.

In experiments where mice are given an intravenous injection of the fungus *C. albicans*, or are given drugs that cause immune suppression, mice may experience symptoms of a systemic infection such as losing weight or symptoms including feeling cold, shivering and lethargy. Mice will lose weight in these models in the first few days of infection without the appearance of other symptoms, or will continue to lose weight alongside the development of 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)?

The majority of mice on this protocol will be either mild (~60%) or moderate (~35%) severity. Some mice may experience severe severity (2-5%).

What will happen to animals at the end of this project?

- Killed

A retrospective assessment of these predicted harms will be due by 1 November 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

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 primarily interested in how antimicrobial drugs affect the composition of the microbiome and subsequent impact on the pathogenic potential of bacteria and fungi which have the ability to cause life-threatening systemic infections in patients who are vulnerable (e.g. those with suppressed immune systems). We are particularly interested in how antimicrobial drugs and microbiome interactions drive the development of antimicrobial resistance and risk of systemic infection. It is not possible to study these questions using in vitro models or humans. In vitro models cannot capture the complexity of the mammalian immune system or its interaction with a microbiome, while analysis of human studies is complicated by additional confounding factors and co-morbidities which are common in the patient population we wish to study. Mouse models are therefore needed to define mechanisms by which antibiotics (and the changes these drugs cause to the microbiome) influence the immune system and development of drug resistance in potential pathogens. This is necessary to understand how common bacteria and fungi, which are normal residents in the microbiome, can become drug-resistant pathogens and develop the insights we need to implement better infection control and clinical



management of these diseases.

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

We have explored the available datasets from humans to analyse some aspects of our research questions. For example, analysis of the microbiome from hospital patients that had been treated with antibiotics and/or immune suppression, or access to databases that hold clinical information about how prior treatments affect susceptibility to infection. We have also run pilot studies using cell lines to examine whether antibiotic treatment of these cell cultures mimic some of the observations we have observed in mice and/or have been observed in patients.

Why were they not suitable?

Clinical databases typically do not have enough patients with the required characteristics to answer our research questions which limits statistical power. For example, many patients who become unwell with fungal infection following antibiotic treatment typically also have additional co-morbidities (e.g. cancer, surgery) which confounds our analysis and makes it difficult to unpick the impact of different treatments/diseases on development of protective immune responses and antimicrobial drug resistance. These patients often have to be excluded from any analysis and this limits the number of patients we are left with to answer our research questions. As such, this approach has not been highly successful so far.

Our initial experiments with cell lines showed that many cell lines do not respond to antibiotics in the same way as primary cells (isolated directly from mice), and that primary cells are not always able to recapitulate what is observed in animal models.

Thus, their usefulness is very limited for these studies.

These models are also unable to model a complex microbiome and immune system, meaning that we are unable to study the intersection between these systems in the context of antibiotic treatment and infection which is the main aim of our studies.

A retrospective assessment of replacement will be due by 1 November 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your experience?

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 mice we require for each experiment based on the expected statistical differences for each type of experiment, and the number of



independent repeats we need to ensure reproducibility of our data. We have worked with these infection models and data types for many years and therefore have good experience with the expected variation and animal numbers needed to achieve our scientific goals. We have also previously sought statistical advice from NC3Rs website and used available tools online (e.g. Experimental Design Assistant) to estimate animal numbers required.

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

We utilise available tools such as the NC3Rs Experimental Design Assistant to ensure our proposed animal numbers in our experiments do not exceed what is necessary to achieve statistically useful results. We also plan our experiments so that multiple tissues are analysed from each individual mouse reducing the total experiments we need to do, but also providing the opportunity to correlate data between different analysis techniques in the same animal. This significantly increases our statistical power and further reduces the number of animals needed across the project.

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

For experiments where the analysis technique is new or the expected variation in data is unknown, we will use pilot studies to determine the expected variation which will help us more accurately determine numbers needed in larger experiments. We will also use correlation analysis techniques in our data analysis to compare data outputs from different tissues in the same animals to enhance our statistical power and further reduce the numbers of animals needed to complete our scientific goals.

A retrospective assessment of reduction will be due by 1 November 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

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 infection models used in this project have been refined by many research groups in the field over the last few decades, and we have further refined the dosage and timing in our own group over the last 5 years. We have previously performed pilot studies to determine the optimal dose of fungal cells for the different infection routes that generate a measurable infection and associated immune response without inducing significant clinical symptoms. We will continue to use these refinements in this project. We will also keep up



to date with new advances in refinements to the infection models as they are published, and implement where appropriate.

We will use mouse models of infection and colonisation for our studies. Colonising mice with potential pathogens causes little distress to the animals since the potential pathogens we propose to study are common inhabitants of mammalian guts under healthy conditions. In experiments where we model an infection, either by injection of the microbes or by disrupting the immune system, mice may develop clinical symptoms of the infection which largely mimic what is observed in patients. We have significant prior experience with these models and have been able to identify infection doses and protocols that limit the development of symptoms while still allowing us to study activation of immune responses and pathogen colonisation. We also have further refined the model with the use of enhanced monitoring steps and implementing extensive clinical scoring sheets to gather additional data in our experiments.

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

We require adult mice for our experiments as they have a fully developed intact immune system that can be manipulated or changed with our drug treatments to mimic what occurs in hospitalised patients. Less sentient animals are not useful here, because they have simpler immune systems that lack many components of the immune system found in humans and we are therefore unable to make meaningful discoveries with potential clinical applications in these other model systems. We are unable to use anaesthetised animals for our studies due to the length of the models and requirement for mice to self-colonise with bacteria or fungi via the drinking water in some protocols.

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

Our mice are monitored at least once daily after infection. We may also provide wet mash food to our animals in some experiments to minimise potential weight loss. Our group members and animal facility staff are experienced in the clinical signs of infection for these models, such as watching for changes in weight and behaviour which may indicate beginnings of disease. As outlined above, we have refined our models to minimise these potential outcomes by altering the dosage and timing of infection.

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

All of our procedures (e.g. injections) will adhere to the best practice guidelines published by LASA and LASA guidelines will be referred to for administration of all substances. We will also refer to ARRIVE 2.0, use the NC3Rs experimental design tool and align with PREPARE guidelines in the design of our experiments.

The infection models we will use have been recently published and are included in best practice step-by-step protocols that are also published by our group and others.

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

I regularly receive 3Rs updates from local mailing lists and email alerts which disseminate information about new models, protocols and upcoming 3Rs-focused meetings. I also set up 'google alerts' for new research of interest that is then directly emailed to me, which



helps me keep up to date with new research and model refinements as they are first announced.

A retrospective assessment of refinement will be due by 1 November 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?



8. Bacterial and host interactions in tuberculosis infection

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

Tuberculosis, Host factors, Bacterial genetics, Antibiotics, Host-directed therapies

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

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

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 study immune and bacterial factors implicated in mycobacterial infections and understand how therapies targeting the host or the bacteria help to control disease.

A retrospective assessment of these aims will be due by 9 November 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?



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?

Tuberculosis (TB) is caused by the intracellular pathogen *Mycobacterium tuberculosis* and remains in the top 10 causes of deaths in low-income countries and worldwide is the 2nd leading infectious killer. In 2021, an estimated 10.6 million people become ill with TB and 1.6 million people died from TB and there were 2.2 million new cases. TB continues to be the leading cause of deaths in HIV infected individuals, killing almost 0.19 million in 2021 (WHO 2023 TB Report). This situation is further exacerbated by the rise in multi-drug resistant (MDR) and extensively drug resistant (XDR) TB strains. In 2020, there were 470,000 cases of MDR TB/RR-TB (Rifampicin resistant TB) and approximately 40,000 of these were XDR. The highest concentration of MDR TB cases occur in India, China, and the Russia Federation. Undoubtedly, new vaccines and antibiotics are required to combat this disease.

Animal models of tuberculosis have made advances in these areas possible. The host response to TB involves the combination of a complex network of host-pathogen interactions. Mainly, the hosts status (genetic resistance, nutritional and vaccination status etc.), environmental factors e.g. coinfections and the virulence of the pathogen. Much of the biology and mechanism of the immune response against *M. tuberculosis* remains unclear. This work helps to assess potential factors in the host and the organism that may contribute to our understanding of the host and pathogen interactions and aid to develop future interventions.

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

This project could result in development of new analysis methods for assessing TB in infected mouse lungs, leading to new discoveries on the importance of host and/or bacterial factors in TB infection/disease and publications from the data obtained using this license. This could result in the developments of new therapies in TB and other diseases. A shorter treatment period is important to increasing compliance of patients taking anti-TB drugs. This ultimately will lead to reduced transmission, lower chance of developing resistance and fewer human deaths.

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

In the short-term the data obtained will be used to develop and inform the groups' projects. Publications from these could benefit other researchers in the area and provide new knowledge with the mid-term potential to enable better understanding of mycobacterial infection and host response. In the long-term, during and beyond this PPL, these findings could impact on TB vaccine and drug development.



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

Where appropriate we will collaborate with groups that have new technology, we can use to investigate samples that can then provide more information for us and them. Data is discussed at internal, national, and international meetings and new discoveries published in open access journals.

Species and numbers of animals expected to be used

- Mice: 25000

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 a range of ages for breeding genetically altered (GA) mice as this requires stages from embryo to adults. We only use adult mice for our experimental work as a mature immune system is required for studying the complexities of the host response and interactions with mycobacteria.

We use the laboratory mouse as the model organism as this is the best-characterised model for these studies, with many features applicable to human tuberculosis infection. Their immune responses are well defined and the technology, enabling sophisticated manipulations of the generation of blood cells and immune system, is highly developed. Mouse transgenic and knockout techniques are well established and fully established; mice have a relatively short generation time; its immune 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. Much of the research in the field of tuberculosis (TB) has been produced from mouse TB studies, driving and supplementing developments in knowledge of infection, the host response, vaccine and drug development. To our knowledge, no other species of lesser sentience can fulfil the requirements of this project to the same extent as the mouse.

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

Typically, mice may be injected or orally administered with a potential immune or bacteria modulating substances such as drugs (from pre-clinical trials or clinical, FDA-approved) e.g. Bedaquiline (an antibiotic used for treating TB) then assessed 2-6 weeks later to define if the substance has altered immune response or improved bacterial clearance. The route by which an immune modulator will be given will depend on which internal organs need to be targeted and this will be done following guidance provided by the developers of the modulator. It is expected that this should be a mild experience with no more than transient pain with no lasting harm. Some modulators may require multiple administrations,



mostly done within dosage and frequencies permitted, to induce a change in the host (mouse). Rarely increased frequency of administrations may be required, for modulators that have a short half-life, using the least invasive route applicable for the specific modulator. These modulators may be used in mice either before or after infecting with mycobacteria which are pathogens that cause TB. To assess for a change in response in these infected mice, samples (e.g. organs) would be taken from humanely killed mice at set time points after infection with studies lasting up to 2 months. Other studies may involve assessing the effect of the impact of a change in mycobacterial genetics on the development of infection in mice with studies lasting up to 4 months. Depending on the type of study, blood samples may be taken from live mice during the course of the study to inform if specific changes are occurring. Also live mice may be examined by imaging techniques to assess for the distribution of modulator and or mycobacteria or for monitoring changing responses to modulation and/or infection.

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

Mice given modulators alone are not expected to display any physical or behavioural changes. However, some strains of mice may be more sensitive than others. If any weight loss and altered general condition is observed e.g. hunched posture or piloerection, this would be monitored, and if this does not resolve within 24 hours then the affected mice will be humanely killed.

For TB infection studies we utilise a low dose infection model which generally results in mild infection in 95% of those infected, normally with minimal weight loss and no symptoms of disease over the duration of our studies. Some strains of mice may be more sensitive to infection. These strains are studied for a shorter time period which should result in no more than moderate severity i.e. less than 10 % weight loss from previous weight checks in combination with changes in body condition e.g hunched posture or piloerection, or 15% weight loss seen in isolation.

There are rare occasions (no more than 5% of infection studies), where more pathogenic strains of mycobacteria need to be assessed. This could lead to moderate severity with up to 15% weight loss from their pre-treated weights. In a very small subset of these mice infected with more pathogenic mycobacterial strains some mice may experience severe adverse effects with potential symptoms i.e. hunched posture, altered breathing, piloerection, subdued behaviour/ lack of movement and up to 20% weight loss from pre-treated weights. If mice display these symptoms 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)?



Overall in the protocols collectively, we anticipate 15% of the mice may have subthreshold severity, 79% may have mild severity, 5% may experience moderate severity and 1% potentially severe severity.

What will happen to animals at the end of this project?

- Killed

A retrospective assessment of these predicted harms will be due by 9 November 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

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?

TB manifests mainly in the lungs of infected individuals involving a complex interaction of various cells with the pathogen. To effectively study this, particularly when examining immune modulators and modified mycobacterial strains, the project requires a complete functional immune system therefore it is impossible to completely replace the use of animals for such studies.

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

In vivo studies will be supplemented by in vitro and ex vivo study results e.g., bacterial strains will be assessed for growth profiles in culture to define strains of interest prior to further analysis in vivo and immune modulators where possible will be optimised in vitro prior to in vivo use. The group is also developing human stem cell-derived lung on a chip (LoC) technologies using differentiated human stem cells aiming to mimic some of the complexity of the host and pathogen interactions. When the system is fully developed, we aim to use these LoC as a predictive tool to replace mice used for infection experiments.

Why were they not suitable?

Assessing bacterial strains and modulators with cells in cultures can provide baseline information and reduce some in vivo studies. To effectively study TB infection, particularly when examining immune modulators and modified mycobacterial strains, requires a complete functional immune system therefore it is currently impossible to completely replace the use of animals for such studies.

A retrospective assessment of replacement will be due by 9 November 2029



The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your experience?

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?

Cryopreservation of gametes, embryos, tissues, and cells will ensure that the minimum number of mice is bred. In addition, we aim to manage the breeding colonies with the least amount of surplus possible given the constraints of study requirements.

For experimental work we calculate the number and types of studies we are likely to perform over the 5 years. Then for this the number of animals required based on our extensive experience of the mouse model and where appropriate, or required, information obtained from collaborators and relevant literature.

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

Initial checks on bacterial strains and immune modulators where applicable will be assessed in vitro. For new modulators, small pilot studies (in our immunomodulation protocol which is done without infection) may be performed which help define the power of the study and therefore potentially reduce animal numbers. Our experimental design takes into consideration the NC3R guidelines and use of the experimental design assistant. For most of the quantitative experiments, design will be based on PREPARE guidelines and sample sizes may be set using power analysis, generally using a significance level of 5%, a power of 80% and at least practicable difference between groups of 20%.

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).

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 with the least amount of surplus possible given the constraints of study requirements. Pilot studies will be performed in our immunomodulation protocol to enable optimisation of experimental conditions. From each mouse we aim to use multiple tissues/organs where relevant to the study. Also, where appropriate we can provide tissues for other researchers/collaborators under the tissue sharing scheme. The



potential to use imaging to assess infected mice would also, in time, reduce numbers required for infection studies.

A retrospective assessment of reduction will be due by 9 November 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

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 mouse aerosol TB infection model will be used in this project. The mouse model is best characterised for these studies, with many features applicable to human infection. To our knowledge, there is not a species of lesser sentience that can fulfil the requirements of this project. Using an aerosol infection model is the most physiologically relevant method for analysing TB infection as TB manifests in the lungs of individuals that have inhaled the organism. A low dose infection is used which results in most cases in mild disease in mouse strains that are relatively resistant to infection. In some cases, depending on the genetic background of the mouse strain and the bacterial strain, severe disease can develop in a subset of mice exposed to more virulent mycobacterial strains. Severe endpoints may be required to enable assessment of strains to mirror the pathogenicity that occurs in humans with clinical strains. To reduce suffering, where the trajectory of the disease can be predicted from previous studies, we aim to perform shorter studies. We will also implement a clinical scoring system with increased weighing of animals progressing up to daily weight checks.

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

The mouse model is best characterised for these studies, with many features of the immune response applicable to human infection. To our knowledge, there is not a species of lesser sentience that can fulfil the requirements of this project. Adult mice are used for these studies as a mature immune system is required to control the infection and to enable assessment of the complexity of the interaction between host and pathogen.



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

Mice displaying any signs of adverse response will have increased inspections and more regular weight checks at least twice per week, progressing to daily if/when necessary. Infections are performed mostly via aerosol that is not an invasive procedure. Most of the adverse responses are in the infected animals and in cases where we suspect enhanced virulence or altered immune responses we will monitor weight and behaviour daily.

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) 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?

TB infection requires a complex interaction of multiple cell types to respond to infection which is hard to reproduce in vitro. New developments of lung on a chip (LoC) technology are promising and this, as well as other new developments in replacement strategies will be continuously monitored. This is currently achieved by constantly reviewing the literature, getting information from the NC3Rs webpage, the NORECOPA (Norway's National Consensus Platform for the advancement of the 3 Rs) and the NIO newsletter. Our group is establishing human inspired organ on a chip (LoC) technology that mimic human tissues (e.g., the alveolar space) in a more physiologically relevant way. This system will substantially contribute to the 3Rs strategy of our group.

A retrospective assessment of refinement will be due by 9 November 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?



9. The impacts of the tumour microenvironment on cancer development, anti-tumour immunity, and therapeutic responses

Project duration

5 years 0 months

Project purpose

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

Key words

cancer, stromal cells, progression, immune reaction, therapy

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

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

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

What's the aim of this project?

To investigate the influences of stromal cells (non-cancer cells within the tumours) on malignant tumour development, anti-tumour immune responses, and the effectiveness of anti-tumour therapies.

A retrospective assessment of these aims will be due by 8 November 2029



The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

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?

Breast cancer and ovarian cancer are the prevalent cancer among women, and prostate cancer is one of the most common cancers among men. While survival rates for the early stages of these cancers have exhibited improvement, the late-stage cases not only pose a life-threatening risk but also significantly affect the reproductive health and overall quality of life of patients. Although surgical removal is often possible at the primary site of cancer, the problem lies in the tendency of late-stage cancer to spread to different parts of the body, a phenomenon known as metastasis. This metastatic spread is an important factor contributing to the high mortality rate associated with cancer. Although metastatic tumours are primarily treated with chemotherapy, the overall survival rate for patients with metastatic tumours has seen little improvement over the past two decades. This suggests that current medications are not effectively addressing metastatic disease, highlighting the imperative need for the development of new therapies to achieve more effective cures.

It has been known that tumours comprise not only cancer cells but also a variety of non-cancer cells, referred to as stromal cells. Previous studies conducted by our lab and others have indicated that distinct types of stromal cells, such as tumour-associated macrophages and cancer-associated fibroblasts, play a critical role in providing essential support for cancer cells in establishing malignant tumours. Furthermore, our research and that of others have demonstrated that these stromal cells are involved in impairing anti-tumour immune responses and contributing to resistance against chemotherapy treatments. These findings strongly suggest that stromal cells within the tumour microenvironment represent novel and promising therapeutic targets. Nevertheless, the molecular mechanisms behind these tumour promoting functions of the stromal cells are still not fully understood.

This project aims to understand the interactions between cancer cells and stromal cells in the context of tumour progression and metastasis. It also aims to identify key stromal factors that influence cancer cell behaviour, impact anti-tumour immune responses, and determine the effectiveness of current therapeutic treatments. While our primary emphasis is on the malignant cancers that impact reproductive health, we will also explore other types of cancer to evaluate the universality of our discoveries. The results of this project will lay the foundation for the development of innovative therapies to combat malignant tumours by targeting crucial stromal factors. Furthermore, the data from this project will



significantly contribute to develop novel approaches for predicting disease outcomes, assisting the selection of the most suitable therapies for this lethal disease. Consequently, this project has the potential to greatly improve the survival, quality of life, and productivity of patients with malignant tumours.

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

This project will advance our understanding of the roles of tumour-associated stromal cells (e.g., macrophages and fibroblasts) in the malignant tumour development, T cell- and NK cell-mediated anti- tumour immune responses, and therapy resistance. Additionally, it will identify novel targets to enhance current therapies for malignant diseases. The data generated by this research will make a significant contribution to the development of effective therapeutic approaches against malignant tumours, as well as novel strategies for early detection and diagnosis of cancer.

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

This project has the potential to benefit a wide range of stakeholders. Researchers in tumour immunology field directly benefit from the project's insights and data, which can advance their understanding of the tumour microenvironment (short-term). Direct beneficiaries also include cancer patients and healthcare professionals who may gain access to more effective and targeted therapies, particularly in the emerging field of immunotherapies (long-term). Indirect beneficiaries encompass researchers studying non-cancer diseases, especially diseases in which cytotoxic lymphocytes play a role via currently unknown mechanisms (short-term). Pharmaceutical companies and biotechnology firms may find opportunities to translate these findings into potential therapeutic products or technologies (mid-term).

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

Results from this project will be disseminated through publications in open-access journals and presentations at scientific conferences. Based on the outputs from this project, we will explore collaborations with other research groups, clinicians, and pharmaceutical companies. Findings will also be communicated to the public through the media, social-media and public lectures, which may attract more public interests in cancer research field. Data will be available to all researchers upon request.

Data suitable for sharing (e.g., transcriptomic data) will be deposited to public databases.

Species and numbers of animals expected to be used

- Mice: 13,000

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.

Most studies have traditionally utilised mice for in vivo models of cancer. Consequently, employing mouse models offers the advantage of utilising insights provided by existing literatures to reduce sample size and refine procedures. The mouse model enables the analysis of the roles of target genes (or cells) using established genetic engineering techniques. Given the objectives of this project, we will primarily use adult mice with mature immune systems. However, in certain cases, treatments will commence during the juvenile stage for specific purposes, such as analysing the tumour development process or conducting bone marrow transplantation.

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

We will **induce primary and/or metastatic tumours** in mice through either genetic engineering (“spontaneous” tumour models) or tumour injection (“experimental” tumour models). In the latter model, animals will be injected with cancer cells (or tissue fragments) through one of the following routes: subcutaneous, intravenous, intra-peritoneal (no anaesthesia) or intra-mammary fat pad, intra-cardiac, intra-splenic (under anaesthesia). On rare occasion (less than 5% of experimental tumour model), the **primary tumours will be surgically resected** (under anaesthesia), and the tumour-resected mice will be maintained until they develop distal metastasis. This procedure will be performed only once for each animal. During the experiment period, tumour growth in mice will be monitored by calliper (no anaesthesia) or non-invasive imaging (under anaesthesia). Before and/or after the tumour development, the animals will receive one or combination of additional treatments depending on the purpose of experiment. Most typical treatment is the **administration of substances** that modify stromal cells, such as antagonists, agonists, blocking antibodies. In rare cases, the **adoptive transfer of stromal cells** (e.g., monocytes) will be conducted on the tumour-bearing animals. To investigate the impact of stromal factors on anti-tumour therapies, animals will be **injected with therapeutic agents** (e.g., drugs used for chemotherapy) or **cytotoxic lymphocytes** (T cells or NK cells). The substances and cells will be injected through one of the following routes: subcutaneous, intravenous, intra- peritoneal, intra-tumoral, or oral gavage (no anaesthesia). In some studies, small volume (less than 100 μ L) of **blood will be taken** from the superficial vein of mice. In rare cases (less than 2% of tumour models), mice will receive **bone marrow transplantation** before the tumour induction to reconstitute immune cells. In this case, mice will receive lethal irradiation (9Gy) or intra-peritoneal injection of Busulfan and will be transplanted with bone marrow cells through the tail vein.

In a typical experiment, individual animal will experience tumour induction, tumour growth monitoring, administration of substances, and blood sampling. In some experiments, administration of therapeutic agents or cytotoxic lymphocyte will be added to these procedures. The most complex experiment could typically involve bone marrow



transplantation, tumour induction, tumour growth monitoring, primary tumour resection, adoptive transfer of stromal cells, and blood sampling. This would be conducted in a maximum 2% of animals. All animals will receive the highest standard of care, and will be provided appropriate social, environmental, and behavioural enrichment.

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

Most animals will develop tumours within one of the following sites: subcutaneous, lung, peritoneal cavity, mammary gland, bone, or liver. The tumour induction will cause long-term distress similar to cancer patients. While duration of experiment differs among the models, it is typically 28 days (experimental model) or 150 days (spontaneous model). The tumour-bearing animals will be regularly monitored and euthanised before showing significant clinical signs, such as abdominal distension, hunched appearance, inappetence, difficulty of breathing, ruffling of the coat, reduced activity. Mice received surgery are expected to recover quickly and will be given painkillers and post-operative care just like people recovering in hospital. Administration of substances and blood sampling may cause transient pain and sometimes result in itchy skin, while it is not expected to cause lasting harm. The bone marrow transplantation has a risk of anaemia and diarrhoea if it failed. Although we are familiar with this method, signs for anaemia (pale toes) and diarrhoea will be regularly monitored. The bone marrow-transplanted mice as well as immune compromised animals are susceptible to infections.

These mice will be maintained in individually isolated cages, administered with antibiotics, and monitored clinical signs.

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

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

Sub-threshold: breeding and maintenance of GA mice; 60%

Mild: breeding and maintenance of GA mice; 5%

Moderate: experimental tumour model (subcutaneous & mammary tumours); 10%

Severe: experimental tumour model (metastatic tumours) & tumour-developing GA; 25%

What will happen to animals at the end of this project?

- Killed
- Used in other projects

A retrospective assessment of these predicted harms will be due by 8 November 2029



The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

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 for understanding how tumour-associated stromal cells influence the malignant tumour development, anti-tumour immune reactions, and the resistance of cancer cells to anti-tumour therapies. These processes occur within the tumour microenvironment that is very complex structure with many different cell types interacting and communicating with each other. In addition, these stromal cells interact with different partners at different stages of tumour development. 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 have conducted literature research and developed several in vitro assays utilising 2D or 3D co-culture systems. These assays aim to replicate specific aspects of tumour-stromal interactions, including stromal cell-induced extravasation (infiltration of cancer cells through blood vessel), immune suppression, and the growth of tumour spheroids. Using these in vitro models, we aim to identify robust candidates among stromal factors that merit investigation in animal models. Concurrently, we will employ these assay systems to validate our findings from mouse models under more controlled conditions. Consequently, these assays serve as valuable tools in substantiating our working hypothesis and identifying key molecules that govern the pro- and anti-tumour functions of stromal cells.

Why were they not suitable?

The currently available in vitro models are over-simplified and do not recapitulate the complex tumour microenvironment where various types of stromal cells (e.g., immune cells, endothelial cells, and fibroblasts) are interacting each other and with cancer cells. We thus need to integrate our in vitro assays and in vivo experiments proposed in this license. Without the use of relevant animal models proposed here, we cannot achieve our research objective.

A retrospective assessment of replacement will be due by 8 November 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your experience?



Reduction

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

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

Number of animals for breeding (Protocol-1) was estimated based on our previous records. Offspring with genotypes that do not meet our experimental purpose (75 - 87.5% of offspring in this protocol) will be euthanised when their genotypes are determined (usually before the age of 4 weeks) and will not be used for experiments. This is unavoidable because this study requires the use of female mice carrying the compound mutation, despite our careful design of the most efficient breeding strategy.

Numbers of animals for experiments (Protocol-2 &3) were estimated using statistical analysis software (e.g., G*Power, Openepi, PWR Bioconductor R Package). We calculated minimum numbers of animals to be used whilst ensuring that the results are statistically significant. Sample sizes for our experiments are estimated from our own earlier studies and published studies conducting similar experiments.

Calculations typically show that we need at least 9 mice per group to achieve the quality of results we need. We have also referred our annual return of procedures data to estimate the number of animals that we will need to use for breeding and experiments.

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

We employed the NC3Rs' experimental design guidance and experimental design assistant (EDA) to plan our experimental design, practical steps and statistical analysis utilising the advice and support for randomisation and blinding, sample size calculations and appropriate statistical analysis methods. We will use the EDA diagram and report outputs to support experimental planning with animal users.

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

We always use the most efficient breeding strategy to generate GA mice with required genotype for experiments, minimising the surplus of animals with an unusable genotype. To determine tumour loads in the internal organ, we use non-invasive in vivo imaging techniques. These methods enable to measure tumour growth in the same animal without euthanising separate cohorts of mice at different time points. To modify stromal factors using non-tumour GA mice, we will transplant the bone marrow from GA to recipient mice. This method enables to obtain a large cohort of mosaic mice in which immune cells



contain the genetic alteration without extensive breeding of GA mice. At the end of the experiment, we will harvest as many tissues as possible at post-mortem. As needed, these tissues are sectioned and used for different experiments. If immediate analysis is not required, we will freeze the tissues or embed them in paraffin blocks, making the samples available to other researchers working on similar questions.

A retrospective assessment of reduction will be due by 8 November 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

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.

Tumour models: To investigate the process of tumour progression, we use genetically altered (GA) mouse models that naturally develop tumours. For such "spontaneous" tumour models, we will mainly use MMTV-PyMT mice. This model develops mammary tumours within shorter duration compared to other GA mouse models, enabling to minimise the duration of distress. We also utilise "experimental" tumour models (i.e., mice injected with cancer cells or tumour fragments) to investigate the late-stage tumours, metastasized tumours, and tumours established by human cancer cells. The proposed models enable tumour development within short-term under well-controlled conditions. We have over 10 years of experience with these models and are familiar with the dynamics of tumour formation.

Based on our experience and published literature, we have determined humane endpoints for each model that are sufficient to answer the scientific question and minimize animal suffering. We will ensure that animals are maintained in the best physiological state by regular monitoring and proper housing.

Surgical techniques: A skin incision is required to remove the primary tumour. These methods allow the tumour development to be restricted to certain areas. A skin incision is also necessary for injecting cancer cells into a specific area of the mammary fat pad, facilitating the restriction of tumour development to designated regions (less than 20% of mammary fat pad injection). In experiments that do not require such precise injection, non-



invasive methods are used. For splenic injections, a small laparotomy is performed to expose the spleen aseptically, avoiding unnecessary leakage of cancer cells into the abdominal cavity. The surgical techniques are well established and cause minimal damage to the animal. Based on our experience, all steps are usually completed within 15 minutes and the animal recovers quickly.

Administration of substances: We will select minimally invasive method depending on the purposes. Intravenous injection, intra-peritoneal injection, or oral gavage will be selected to achieve rapid systemic distributions of agents. Subcutaneous injection or intra-tumoral injection will be chosen for more localised or slow distributions. The dosage and frequency that do not cause serious side effects are determined based on our experience and published research.

Non-invasive imaging: This is the most reliable and least harmful method to detect the tumour growth in the internal organs. Imaging is performed under anaesthesia and completed in a short time (typically within 15 minutes), minimizing distress on mice.

Blood Sampling: This is essential to validate efficacy of the stromal modification and to investigate the effects of stromal modification on numbers and phenotype of circulating immune cells. Blood sampling from superficial blood vessels can minimize pain and suffering.

Bone marrow transplantation: This is a useful refinement technique to restrict the genetic alterations in hematopoietic cells. Lethal irradiation is essential to achieve complete depletion of existing bone marrow cells and thereby achieve high reconstitution rate. With busulfan, the risk of mucosal damage and infection is reported to be lower than irradiation. Therefore, in studies that do not require complete bone marrow replacement, busulfan will be given to recipient mice instead of irradiation.

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 or their immune system is too different from the human immune system to provide relevant results. We can not use embryos or very young animals as their immune system is immature and doesn't respond to antigenic stimulation in the way mature animals do.

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

Tumour models: By intrasplenic injection, cancer cells will disseminate to the liver as well as spleen. To restrict tumour development in the liver, the spleen will be resected after tumour injection. Although intra-cardiac tumour injection is the most common and reliable method to develop bone metastasis, a recent study has proposed injection of cancer cells into the caudal artery in the tail. We will thus test this route in a small cohort of mice under consultation with the NVS. If it works well, we will use it as an alternative and more refined



method to investigate bone metastasis. In spontaneous tumour models, expression of oncogenes is regulated by specific promoters (e.g., MMTV promotor to restrict PyMT expression within mammary gland epithelial cells), which limits the site of tumorigenesis. Based on published and our previous data, we have determined maximum acceptable tumour loads that enable to achieve our experimental aims as well as humane handling. To keep animal suffering minimum, we will regularly monitor tumour loads by calliper and/or non-invasive imaging. The tumour-bearing mice will be regularly monitored for clinical signs, and euthanised before they display them. Typically, the clinical signs to be monitored include hunched appearance, inappetence, ruffling of the coat, difficulty of breathing, reduced activity, abdominal swelling, and lymph node enlargement.

Surgical techniques: The procedures requiring surgery (e.g., splenic injection and subcutaneous transplantation of tumour fragments) will be performed on a limited number of mice as the project progresses. Pain will be controlled during surgery by general anaesthesia and peri-operatively by analgesics. Bleeding during surgery will be controlled by cautery or applying local pressure. Risk of infection will be minimised by good surgical and aseptic techniques. Surgical and implantation sites will be monitored for signs of inflammation and infection (e.g., hunched appearance, inappetence, difficulty of breathing, ruffling of coat, reduced activity).

Administration of substances: All substances will be administered at doses known to be non-toxic, which was determined based on our experience and published studies. Administered volumes will be determined in accordance with guidelines. Body weight, behaviour (e.g., lack of grooming), and coat condition of animals receiving substances will be regularly monitored. Gavage administration will be conducted only by experienced handlers using aseptic technique, fine gauge needle, good restraint, and slow delivery of small volumes. When substances in drinking water are assumed to reduce palatability, 5% sucrose will be added to the water. For anti-cancer therapeutic agents, doses will be determined based on clinical regimen and published animal studies and will not exceed the maximum tolerated dose.

Blood Sampling: Dilation of the vein by warming and local pressure to stop bleeding will be carried out. To avoid hypovolemia or anaemia, no more than 10% of the total blood volume (TBV) will be drawn on any one occasion and no more than 15% TBV in any 28-day period.

Bone marrow transplantation: To minimize the risk of infection, the animals will be administered antibiotics and maintained in IVCs under barrier conditions. Animals received bone marrow transplantation will be monitored for signs of anaemia (pale toes), diarrhoea, and above-mentioned clinical signs. Animals will be euthanized when they begin to exhibit one of these signs.



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

All animal experiments will be conducted and published following the ARRIVE guidelines as well as PREPARE guidelines.

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 will regularly check information on NC3Rs website. We will also attend regional 3Rs symposia.

A retrospective assessment of refinement will be due by 8 November 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?



10. Treating cholinergic crisis

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

Cholinergic crisis, Treatment, Acetylcholinesterase

Animal types	Life stages
Guinea pigs	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

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 new treatments for exposure to cholinesterase inhibitors (ChEIs).

A retrospective assessment of these aims will be due by 2 November 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?



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?

Cholinesterase inhibitors (ChEIs) are in widespread use in society for various purposes. These compounds are highly toxic and it is estimated that ~250,000 people die every year because of accidental or deliberate ChEI intoxication. Treatment can be difficult and protracted depending of the ChEI and the route of exposure. More effective approaches are needed to treat exposure to these compounds.

ChEIs interfere with the nervous system. They stop the normal function of the enzyme acetylcholinesterase (AChE) which results in the build-up of the neurotransmitter acetylcholine (ACh). If there is a sufficient build-up of ACh, the muscles and organs controlled by ACh stop working properly and a cholinergic crisis occurs, which affects multiple body systems. The effects of cholinergic crisis include miosis (constriction of pupils), excessive secretions (e.g. saliva, tears and sweat), muscle tremors and convulsions. Acute, serious effects include seizures and respiratory failure which can cause death or lasting damage.

Currently available treatments are not totally effective and do not address all the body systems that are impacted by ChEI exposure. Improved treatments could increase the survival and long-term prognosis of those exposed. Work under this licence will assess new compounds for improving the treatment of ChEI exposure.

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

The main output will be new knowledge from assessments of new treatments for ChEI exposure. This will be compared to knowledge of existing treatments to understand and realise the benefit these new treatments may provide.

In the short term, work planned under this licence will contribute to scientific knowledge by identifying therapeutic targets and mechanisms to further our understanding of the toxicology of ChEIs and its mitigation by treatment. The initial focus of the project will be treatments that preserve the function of the respiratory muscles, where failure of contraction has life-threatening effects. The combination of cell-based assays, animal tissue assays and live animal work on this licence will enable a comprehensive assessment of new drugs and/or combinations of drugs to treat exposure to ChEIs and will build on work conducted under a previous licence. Unlike the development of many other drugs, there are limited opportunities to test new treatments in a controlled clinical population so information gained from animal studies is critical for the development and licensure of these drugs for human use. We will publish our results, where appropriate, to ensure that other researchers in this field can benefit from our findings.



In the longer term, work conducted under this licence to identify new approaches to treat ChEI exposure, is expected to drive regulatory development of new treatments. This is then expected to provide clinicians with more effective treatments for those exposed to ChEIs. This will increase the treatments available to clinicians treating people exposed to ChEIs.

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

This work will produce underpinning data that will inform and guide future research directions for researchers and clinicians in this field. It will generate data to position candidate compounds for further development as improved treatments for ChEI exposure. However, work under this licence is early stage research, and, as such, is not likely produce a licenced improved treatment during its lifetime.

In the longer term, if a future treatment identified from this work is licenced, it will provide benefit to those exposed to toxic doses of ChEI.

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

Results (both positive and negative) will be published in the open literature, at conferences or in internal organisational records. Data will be shared with other researchers with similar interests including companies, academics and international or national scientific partners. Outputs that are not published are recorded in the organisation's information management system as technical reports.

These will be shared with scientific partners through formal collaboration to ensure that this knowledge is appropriately and effectively used to advance the aim of this work.

Species and numbers of animals expected to be used

- Guinea pigs: 780

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 guinea pigs will be used in all the protocols on this licence.

To do the planned work we need to use animals that have a response to ChEI exposure similar to that of humans: guinea pigs match this criterion, they are the preferred species for efficacy experiments based on their biochemistry, size and the prior information that is available on their response to ChEI exposure and treatment. Guinea-pigs are also amongst the lowest-order mammalian species suitable for this work and are therefore used in all protocols in this project for consistency.



Guinea pigs have levels of enzymes that bind ChEIs in the blood that are the most similar to those in humans, and, of the small laboratory animal species, guinea pigs best predict the responses to treatments seen in non-human primates and by extrapolation, in humans. The size of guinea pigs also allows for multiple blood samples to be taken from individual animals in studies that monitor the level of compounds in blood over time. Adult guinea pigs have been used extensively to investigate ChEI exposure and therapy compounds. Pre-existing data in the adult guinea pig will inform our experimental approaches and guide dosing in the current studies.

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

There are 4 types of study in this project licence.

Study 1: Identification of potential treatments

Functional nerve-muscle tissue preparations will be obtained from guinea pigs for use in experiments to assess whether new treatments are effective, before these treatments are used in living animals. In these studies, adult guinea-pigs will be killed whilst under general anaesthesia, after which diaphragm muscles will be taken and used in experiments in vitro.

Study 2: Tolerability of potential treatments

The aim of these studies is to identify an acceptable range of doses of a new treatment compound for use in subsequent efficacy studies. A range of doses of the treatment will be used, starting with the lowest dose that we believe will be effective.

On the study day, animals will be singly-housed to allow for close observation and monitoring of respiration and temperature. Animals will receive a single administration of the treatment and will be monitored to observe the biological impact that the administration causes. Treatments will be injected into muscles (intramuscular: i.m.), or under the skin (subcutaneous: s.c.). Treatment doses will be incrementally increased until the predicted maximum efficacious dose is given or the animals exhibit minor clinical signs (abnormal movement or transient and minor effects on respiration). No further animals would be dosed at higher levels. Animals will be killed if unacceptable adverse effects are observed at any time. Examples of unacceptable effects include: rapid & shallow breathing or slow belly-breathing for longer than 15 minutes or modest impairment of movement for more than 1 hour. The maximum duration of the study will be 6 hours after treatment injection.

Study 3: Efficacy of potential treatments

The aim of these studies is to determine if candidate compounds can improve the performance of the current life-saving medical countermeasures that are used as immediate therapy for treating ChEI exposure. The majority of animals will be used in these studies.



On the study day, animals will be singly-housed to allow for close observation and monitoring of respiration and temperature. After an acclimatisation and baseline period (≤ 1 hour), animals will be injected once (s.c.) with a ChEI, after which treatments will be injected (i.m. or s.c. – typically one or two injections, but could be up to four spaced out over the study). The animals will then be continuously observed for the effects of ChEI exposure and the reversal or mitigation of these effects by the treatment. Animals will be immediately culled if they meet the criteria for humane endpoint which is substantial and continuing cholinergic crisis with pronounced hypothermia. The maximum duration of the study is 6 hours following ChEI injection, after which the animals will be humanely killed. The timeframe has been set to balance the distress caused to the animals by the effects of the ChEI against the need to generate sufficient scientific data to determine if the treatment can protect against the worst effects of ChEIs for a sufficient duration.

Study 4: Pharmacokinetics of treatments

The aim of these studies is to measure the levels of treatments in the blood. This will assist with the interpretation of efficacy data and enable the optimisation of dosing schedules. To enable humane and reliable blood sampling, surgery will be performed under general anaesthesia to insert a tube into an artery and to attach the tube to a blood-sampling port on the back of the animal. Animals will be given at least 6 days to recover, during which time they will be given appropriate pain medication.

On the study day, animals will either be group-housed or singly-housed in a restricted environment (maximum 7 hours). If they are singly housed, a tube will be connected to the blood-sampling port and kept out of the way of the animal using a counter-balanced tether system. After a baseline blood- sample, animals will be injected with a single injection of treatment (i.m. or s.c.). Multiple blood samples will then be taken. Six hours after the injection, or after the final sample (if sooner) the animals will be killed.

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

The expected impacts will depend on the study type. In studies 2 to 4 the animals may experience some initial and transient distress when they are singly-housed and placed in the restricted space environment.

Study 1: Identification of potential treatments

There are no expected adverse effects as the animals are anaesthetised before being killed.

Study 2: Tolerability of potential treatments



Ideally, a treatment will have no adverse effects. However, some adverse effects will be deemed acceptable, depending on their severity and duration. Expected potential adverse effects of some of the new treatments being investigated include transient respiratory depression and minor problems with normal movement.

The majority of animals are expected to receive doses of treatments that cause no or minor adverse effects. If a treatment does cause minor adverse effects, such as minor impairment of movement, the maximum duration this would be is 6 hours after treatment injection. A six hour timeframe has been selected to match the initial effects of acute ChEI exposure. If an animal shows adverse effects above the threshold of acceptability, the animal will be killed immediately. Examples of unacceptable adverse effects include: rapid & shallow breathing for longer than 15 minutes, gasping or slow belly-breathing, or significant impairment of movement..

Study 3: Efficacy of potential treatments

The primary aim of these immediate treatments for ChEI poisoning is for them to prevent poisoned individuals dying.

There will be adverse effects from exposure to the ChEI. These will depend on both the ChEI, its dose and the doses and effectiveness of the treatments. The adverse effects will range from death (treatment inadequate) to minor, temporary effects (treatment effective). Where possible animals will be prevented from dying from the effects of the ChEI by use of a humane endpoint (HEP see below), however some ChEIs are extremely toxic and their effects occur very quickly, therefore it is possible that animals may die before the HEP can be implemented.

All animals treated after ChEI exposure will experience some initial effects of the ChEI despite the effectiveness of any treatment, due to the speed of action of the ChEI. It is possible that some treatments may only have a short-term effect so animals may improve and then worsen. With treatments that are effective over the experimental duration, animals are expected to recover gradually over time. Previous studies have shown that animals given high doses of ChEI and treatment that is not sufficiently effective may experience more substantial effects such as seizures and loss of movement. It is generally accepted that animals that are seizing are unconscious and therefore unaware. How long the adverse effects persist depends on the timing and effectiveness of the treatment injection(s). In cases where the challenge dose of ChEI is high, and the treatments are ineffective, unconsciousness and death will occur rapidly, with the animals experiencing a limited duration of suffering. The maximum length of time an animal may experience adverse effects for is 6 hours after ChEI injection. A 6 hour timeframe has been chosen as this is a critical period in the medical management of acute ChEI exposure and treatments need to show efficacy over this duration.

Study 4: Pharmacokinetics of treatments



There will be some pain and discomfort following surgery, however, this will be controlled with pain medication and the animal will be given sufficient time to recover before the study. The dose of treatment will be selected based on findings from Study 2 and ideally should have no effects but there may be some minor ones. Typically the dose will also have been shown to have some efficacy in Study

3. The maximum duration of such effects e.g. minor impairment of movement, would be for 6 hours after treatment injection. There should be no adverse effects resulting from the blood sampling.

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: ~ 32 %

Mild to Moderate ~34 %

Severe: ~ 34 %

What will happen to animals at the end of this project?

- Killed

A retrospective assessment of these predicted harms will be due by 2 November 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

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?

ACh is a neurotransmitter in multiple body systems, functions and organs. This means that cholinergic crisis has many different effects, both direct and indirect, throughout the body. To understand the potential benefit of a treatment, we need to study the effects upon the whole system. For example, if a new treatment is able to reduce the effects of a ChEI on respiration, what are the knock-on effects upon the rest of the body? Does this increase survival? Does this decrease the duration of incapacitation? Is the treatment able to reach the target organs in sufficient concentrations to be effective? Are the doses required to do this toxic? Some of this can be studied without the use of animals by looking at individual



systems (and part of this project supports this), however, it is only by understanding the effectiveness of the treatment in a whole living animal that we will be able to confirm the potentially life-saving effects of candidate treatments.

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

As stated above, this work requires the use of a whole animal to assess new treatments. We have considered cell-based systems, tissue preparations and simple model organisms such as *C. elegans* and zebrafish larvae.

Why were they not suitable?

As part of the wider project, cell-based systems and simple model organisms are used to identify treatment approaches, assess their potency and investigate their mechanisms of action. Whilst these studies reduce the requirement for animal experiments and refine the approaches used they cannot replace mammalian animals for determinations of the effectiveness of new treatments.

As stated above, exposure to a ChEI causes a complex and multi-system response. This response can change with ChEI, dose and route of exposure. This project is focused on developing treatments for ChEI exposure and needs to be able to understand the effects of treatments on this complex process, including direct and indirect effects of exposure. Currently cell-based systems and tissue preparations cannot provide the required complexity to understand the response and the potential benefit that a treatment may provide.

Equally, simple model organisms such as the nematode worm *C. elegans*, cannot provide the level of complexity required. Whilst they can model aspects of ChEI exposure their evolutionary distance from mammalian species and humans means that critical aspects of ChEI exposure cannot be investigated. Nevertheless, we are currently investigating their suitability to provide an additional step in the initial identification and assessment of new therapeutic targets. As the ultimate goal is to identify treatments that could be developed into licenced clinical treatments for use in humans, animal data will be required to show effectiveness, the dose required and an understanding of the mechanism of action in a relevant animal species.

A retrospective assessment of replacement will be due by 2 November 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your experience?

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 estimation is based upon the planned work, including the number of treatment compounds and the number of ChEI against which they need to be tested. Previous group sizes used in similar studies and an understanding of the variability of response in the guinea pig have also contributed to the assessment. Input to the design of the planned studies has been provided by statisticians.

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

A staged approach is used throughout the licence Action Plan to select those compounds that are most likely to be effective and acceptable (with respect to safety and tolerability), thus reducing the number of studies and therefore the number of animals needed.

Cell-based screening is used to select the most promising compounds at specific therapeutic targets, thus reducing the number of compounds taken through into testing in animals. The initial effectiveness of compounds is also determined using nerve-muscle preparations that reduce the need for subsequent experiments in conscious animals. Tolerability studies are designed such that the fewest animals are needed to provide the assessment again preventing further assessment of compounds that do not meet tolerability requirements. These approaches will reduce the total number of animals used and reduce the risk that ineffective treatments would be investigated in animals.

The study design has had, and will continue to have, input from statisticians in both the design of the studies and the analysis of the data. This will ensure group sizes are continually considered and optimised throughout the duration of this Licence. Where appropriate, pilot studies and/or interim analysis will be carried out to guide the selection of the correct group size. Where control groups are required, wherever possible and appropriate, these will be shared across multiple studies / treatments. Wherever possible and appropriate, historical control data will be used. An adaptive design will be used in studies measuring dose-response relationships to maximise the characterisation of the relationship using the minimum number of animals.

In studies measuring the concentrations of treatments in the blood (pharmacokinetic studies), the surgical cannulation of animals will allow more blood samples to be taken from each individual animal, which will reduce the number of animals required.

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



Where appropriate, pilot studies and interim analysis will be used to determine the required group size, dose required or timing of samples.

In the nerve-muscle tissue experiments it is possible to test multiple compounds or doses of a compound with each tissue, thus optimising tissue use from that animal and thereby reducing the number of animals required to provide tissues.

A retrospective assessment of reduction will be due by 2 November 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

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 an extensive background knowledge of the animal model (guinea pig) in respect to the effects of ChEI exposure and the treatment of these effects. We understand the benefits and weakness of this model and how it relates and compares to other animal models (such as non-human primates) and to poisoning in humans. We will use this knowledge extensively in this licence to design effective experiments, using existing data as appropriate to provide control data.

Our Action Plan involves staged approach so that candidate treatments progress gradually to those experiments that would be likely to result in the most harm in the absence of an effective treatment. In this way we expect to identify the most effective treatments and this effectiveness acts as a key control of pain, suffering and distress in efficacy studies.

Initially, treatment identification will use cell-based assays, simple model organisms and in vitro nerve- muscle preparations. Nerve-muscle tissue will be taken from animals killed whilst anaesthetised which will prevent the animals from experiencing anything beyond the minor discomfort of the initial restraint during the induction of the anaesthesia. These nerve-muscle preparations allow us to examine treatment effects at their target site following exposure to ChEIs.

Compounds showing effectiveness in vitro and in nerve-muscle tissue will then be assessed to ensure that they do not cause unacceptable adverse effects. These studies



will be carried out in a limited number of animals with an endpoint that has been modified and refined from previous studies and which aims to identify treatments with wide (safer) therapeutic windows.

Effective compounds that are tolerable and safe are then assessed in studies designed to determine if they can improve the treatment of ChEI exposure. These studies use the lowest dose of ChEI that will engage the toxic mechanism against which the novel treatments work. Thus, the experiment is most likely to identify effective treatments and, importantly, is most likely to benefit from the treatment effect having impact to reduce the degree of pain, suffering and distress that the animals will experience. Due to the highly toxic nature of ChEIs and the potential severity of the cholinergic crisis, animals that are exposed to these ChEI doses may likely experience high levels of suffering or distress within one to five minutes of ChEI administration. The effective post-exposure treatments are then expected to improve the animals' condition reducing the harms experienced.

If effective treatments are identified then studies may include increased doses of ChEIs to determine the limits of effectiveness for selected treatments. These doses will produce increasing degrees of harm to the animals with some animals reaching the humane endpoint or dying from effects of ChEI exposure. For some animals this may be very rapid (within minutes) but for others, if the treatment is only partially effective, animals may be incapacitated for several hours.

In all efficacy studies we have identified a need for earlier humane or experimental endpoints to limit harms to the animals. This is a significant challenge and therefore has been addressed by the inclusion, in this licence, of a specific objective to explore the development and application of earlier endpoints. This is backed up by a refinement introduced to the routine monitoring of critical ChEI effects. This will focus on measuring the effectiveness of breathing in the exposed/treated animals, a key mechanism of toxicity for ChEIs and the therapeutic target for the compounds being assessed.

A further refinement that is applied to the studies of treatment effectiveness is a reduction in the overall duration of the study after ChEI exposure. Previously effectiveness has routinely been assessed over 24 hours following exposure, whereas the experimental design here can achieve the same assessment using a 6 hour end point, significantly reducing the duration of potential suffering of animals.

In some experiments it may be necessary to measure treatment drug concentrations over time in the animals. Here, surgical preparation of animals for blood sampling (blood vessel cannulation with a vascular access port) is considered the most refined way of conducting these experiments as it reduces the overall pain, suffering and distress that animals would experience from repeated sampling from superficial blood vessels. The reliability of blood sampling in this way also increases the number of samples that can be obtained from one animal with a corresponding reduction in the number of animals required for the experiment.



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

Species that are less sentient or at immature life stages are not suitable for this work as they do not possess all of the required cholinergic signalling pathways that are required for an effective assessment of survival probability that would be predictive of effects in humans. We have used the simple model organism *C. elegans* in drug and target discovery experiments, but this simple model organism is not suitable for assessment of medical countermeasures for the reason stated previously and because it is not possible to administer doses of treatments effectively and in a controlled manner in this species.

Terminal anaesthesia has been considered for these studies but is incompatible with the scientific aims of the project. Anaesthetics can interfere with the effect of ChEIs and the pharmacological effects of the therapy components. Anaesthetics can inhibit AChE, prevent ChEI-induced seizure activity, act as neuroprotectants and can modulate cardiovascular and respiratory function; effects normally targeted by the current pharmacological treatment of ChEI exposure. As a result, anaesthetics have been shown to produce significant survival benefits following ChEI exposure. Anaesthetics have also been shown to interact with 1) some of the receptor targets of the candidate compounds and 2) with compounds that have similar mechanisms of action to those of some of the candidate compounds.

Consequently results in an anaesthetised model would be unlikely to be representative of those in a conscious model. The pertinent information that could be gained from studies using terminally anaesthetised animals will be gained from the nerve-tissue studies described above.

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

In all studies, animals will be regularly observed during any baseline period and continuously following administration of their first compound (treatment or ChEI) until the end of the study. Experimental and/or humane endpoints will be in place for tolerability, efficacy and pharmacokinetic studies. During the lifetime of this PPL further efforts will be made on refinement of humane endpoints for animals administered ChEIs. In all efficacy studies we have identified a need for earlier humane or experimental endpoints to limit harms to the animals. This is a significant challenge and therefore has been addressed by the inclusion, in this licence, of a specific objective to explore the development and application of earlier endpoints. This is backed up by a refinement introduced to the routine monitoring of critical ChEI effects. This will focus on measuring the effectiveness of breathing in the exposed/treated animals, a key mechanism of toxicity for ChEIs and the therapeutic target for the compounds being assessed.

Appropriate post-operative care and monitoring (including pain medication) will be employed for animals undergoing surgery.



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

All surgery will be done aseptically following the relevant LASA guidelines.

Available guidelines on the NC3Rs website regarding blood sampling, routes and volumes will be followed. Available guidelines will also be followed regarding the administration of substances.

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

Updates on advances in the 3Rs are regularly distributed by the Named Information Officer. The Licence Holder is actively engaged in the establishment's Animal Welfare and Ethical Review Body (AWERB). Any appropriate advances will be discussed with veterinary staff and, where appropriate and compatible with the scientific aims of the project, these advances will be incorporated. The Licence Holder and all staff working under this licence maintain their required annual CPD, attend relevant external scientific meetings and have meetings and teleconferences with international collaborators working within this field with the aim of identifying, sharing and implementing best practice.

A retrospective assessment of refinement will be due by 2 November 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?



11. The cell and molecular mechanisms of general anaesthetics

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Anaesthesia, Emergence, Astrocyte, Isoflurane, Mitochondria

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

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

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?

While inhaled anaesthetic gasses like isoflurane, sevoflurane, and desflurane (together known as volatile anaesthetics) are widely used in human medicine, the exact mechanisms of their intended and off-target effects are, remarkably, still not well understood. Our goals are to define the cell and molecular mechanisms of anaesthesia and those cell and molecular mechanisms responsible for unintended off-target effects of anaesthetics.

A retrospective assessment of these aims will be due by 16 November 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?



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 their prevalence, the way volatile anaesthetics (gasses like isoflurane) work is still not understood. While these drugs are remarkably safe, a better understanding of how these drugs act is still very important, as they can have significant negative effects in certain groups. One specific area of research we are focusing on is the action of emergence, or 'waking up', from general anaesthesia.

Emergence occurs in thousands of patients daily, but the process is poorly understood and delays in emergence are associated with anxiety, confusion, delays in the operating room, increased risks of injury, and significant medical costs. In addition, in certain groups, emergence does not go smoothly, and long-lasting detrimental effects can result from anaesthesia. For example, geriatric (elderly) patients are at risk of post-operative cognitive decline (loss of cognitive ability, i.e. impaired mental capacities) following general anaesthesia. If the mechanisms of emergence can be understood, it may be possible to develop strategies for active promotion of recovery of consciousness, benefiting patients, doctors, and the general public.

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

The primary outputs of this project will be the advancement of basic knowledge in the fields of anaesthesia, biology, and medicine. The work in this project may lead to translational advances in the use of anaesthesia, but translational developments are not the immediate goal of these studies.

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

We anticipate that researchers, clinicians, and ultimately patients will benefit from these studies.

In the short term, our findings will benefit other researchers in the areas of anaesthesia, astrocyte (a type of cell in the brain which supports neurone function) biology, neurobiology, and mitochondrial biology. They will be important for furthering basic research in these fields, and will increase our understanding of how anaesthesia works.

In the long term, results of this work may benefit patients in certain populations defined by genetics (such as those with genetic diseases like mitochondrial disease or malignant hyperthermia) or age (elderly and children or infants), where anaesthesia is more risky, and knowledge of the mechanisms of anaesthesia may prevent toxic outcomes such as post-operative cognitive dysfunction (loss of cognitive ability, i.e. impaired mental capacities).

As delays in emergence are associated with anxiety, confusion, resistive activity, delays in the operating room, increased risks of injury, and significant medical costs, healthcare systems, and the general public, may also benefit in the long-term.

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



The information gathered will be disseminated to scientists and the general public via publication in peer-reviewed scientific journals and in scientific and public lectures. We will select open-access options for publication to allow for immediate public access without restriction. We will openly share our raw data. We actively collaborate with a number of researchers in our field and will continue to welcome new collaborations.

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.

As we are studying the role of astrocytes (a type of brain cell that supports neuron metabolism) in anaesthetic responses, some work must be performed in living animals. In particular, it is not possible to study the complete process of emergence ('waking up') from anaesthesia in any other setting. Our project will use primary cell culture models for many research questions such as how astrocyte cellular energy and metabolism are cell-autonomously (in the absence of other cell types) impacted by anaesthetics, but some questions, like 'which brain regions house specific cell types important for emergence from anaesthesia' can only be answered using an intact animal.

Brain architecture, anaesthesia responses, and relevant molecular structures (such as components of mitochondrial machinery) are quite different in non-mammalian animals. Non-mammalian models do not contain many brain structures present in mammals, the energy producing components of mitochondria (a component of cells responsible for energy production, signalling, and metabolism) are different (in many cases absent), and astrocyte function is poorly understood but clearly divergent (for example, invertebrates can live without astrocytes, they appear unnecessary in lower organisms). To study the processes that are of relevance to humans, a mammalian model is needed. Mice provide the simplest mammalian model available.

For some experiments, only primary cells (those which are taken from living animals) will be used. For this, only breeding and euthanasia are necessary. These animals are needed as astrocyte cultures can only be generated from cells isolated from mammalian brains. In these experiments, animals will be euthanised for brain cell isolation. Neonates will be used for the generation of primary astrocyte and mixed brain cell lines (these cannot be prepared using older animals, as mature neurons do not survive the culturing methods), while adolescent and adult animals will be used for primary mature astrocyte experiments. Genetically altered animals will be used in order to allow for cell-specific targeting of astrocytes and fluorescent labelling of target cells.

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

For many experiments, only breeding and euthanasia will occur.

For some experiments, animals will be exposed to anaesthesia, or control conditions at between 4 and 10 weeks of age and humanely killed for tissue collection. This does not



include any surgery, as we are studying anaesthesia itself, these animals are only exposed to anaesthetic once.

For the final set of experiments, the typical animal experience will be as follows:

-Animals will have an ultra light-weight, non-reactive, identifier applied at weaning when animals are assigned to groups.

-At the age of 25 days, animals will undergo microinjection into the designated brain regions whilst under general anaesthesia under aseptic conditions.

-At age of 60 days, 35 days after viral injection, animals will be anaesthetised, with the induction (falling asleep) and emergence (waking up) doses measured by gradually increasing then reducing the concentration of inhaled anaesthetic. Induction and emergence will be assessed in different cohorts.

-During the anaesthesia exposure, we will assess induction (falling asleep) and emergence (waking up) doses and timing by gently clamping the tail to see when they respond and tilting animals briefly to their side to see if/when they turn themselves upright.

-At a date shortly after this anaesthetic procedure is performed, animals will be euthanised and their brains collected for tissue analysis.

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

The identifier tag is applied in the ear punch collected for genotyping, and should not result in any additional pain or discomfort. The tags are removable in the event irritation occurs.

Accordingly, we do not expect this will lead to any discomfort or stress.

The microinjection procedure is done under full anaesthesia, with analgesic provided. This has been found to effectively manage pain following this procedure, and the procedure is not expected to result in any lasting pain or other adverse effects. The genetic change (deletion of *Ndufs4*, a gene which encodes for a specific mitochondrial protein, in astrocytes) does not cause any overt phenotypes, at least through the ages we will be studying, accordingly to published studies from our collaborators in this research area. The stereotactic (this means highly accurately positioned) microinjection techniques and viral vector are commonly used. This procedure is expected to cause only moderate stress and mild to moderate discomfort (pain from the procedure will be treated with analgesics), with no lasting harm.

Anaesthesia is a moderate stress, but the tail-clamp (which is used to assess anaesthetic depth, i.e. whether the animals fully anaesthetised) is a non-invasive method which measures spinal cord mediated responses while mice are still unconscious, and loss of righting reflex is a non-invasive assessment of anaesthetic state. Neither will cause significant or lasting pain or discomfort.

Animals are carefully monitored during the surgical procedure (stereotactic injection), and will be monitored for any adverse events using a score sheet developed in collaboration with the animal care team and veterinarians on staff.

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)?



Severe: 1%
Moderate: 24%
Mild: 75%

What will happen to animals at the end of this project?

- Killed

A retrospective assessment of these predicted harms will be due by 16 November 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

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 study is to define the role of a special brain cell type, astrocytes, in the effects of anaesthesia. Some of our experiments are intended to determine which parts of the mammalian brain house astrocytes which participate in the process of emergence ('waking up') from anaesthesia. Since we are trying to identify the parts of the brain necessary for emergence in humans, we need to use a model that contains similar brain anatomy. In addition, the gliotransmitter (astrocyte signals) and neuronal pathways we are studying are partly unique to mammals. Mice are the simplest model which can be used to achieve this goal. An intact, living, animal is needed, because we are assessing actual anaesthesia state - asleep versus awake by two measures which depend on animal behavioural responses. Since emergence involves signalling factors that are only present in living animals, these are necessary.

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

We use cultured cell models whenever possible in these studies. As astrocytes cannot be immortalised, these experiments still require animal use, but simply to collect samples for primary cell isolation.

We have considered alternatives such as organoids (which are clumps of pseudo-tissue grown in culture, typically using cells collected from neonatal mice) and similar ex-vivo (outside of living animal) models such as brain slices in culture.

Why were they not suitable?

It is not possible to study emergence from anaesthesia in a cell culture model, and it is not possible to study the role of astrocytes in emergence in a simpler animal model. Organoid and brain slice models can be used for neuronal activity assays, but these are not viable replacements for assays that assess anaesthesia and emergence. Emergence, in particular, is a process that involves sensory inputs and stimulation through



norepinephrine (aka adrenaline) signalling systems which are not present ex vivo, even if some relevant activity assay existed. In addition, the regional targeting of astrocytes to define which populations (by physical location) are important for emergence is not possible in ex vivo samples

- the region-specific targeting by stereotactic surgery methods requires animals are allowed to grow for some time after surgery. No alternatives to live animal use currently exist.

A retrospective assessment of replacement will be due by 16 November 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your experience?

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 use published and preliminary data to estimate variance within groups, and to judge what effect sizes would be biologically significant. We use the key endpoints in individual studies which are limiting factors (based on variance, relevant effect sizes) to determine replicate numbers necessary.

Our estimates include both male and female animals to ensure that our data are not sex biased or unable to detect significant sex differences that may be present.

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

During design phases of this project and project licence, we referenced the NC3R's and other relevant regulatory requirements, as well as discussing our experimental strategies with statisticians and online statistics and experimental design references. Experiments are designed to minimise the number of control groups. Good experimental design also minimises variables which could increase variance between individual animals in a given treatment group, a consideration which not only increases quality, but also reduces the number of animals needed to detect differences.

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 store catalogued tissues and other biological samples not immediately used and will make these available to other researchers. This provides a potential avenue for reducing the need for additional mice in studies conceived at a later date by us or other groups.



A retrospective assessment of reduction will be due by 16 November 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

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 experiments probing the populations of astrocytes responsible for altered emergence from anaesthesia, animals undergo one brain microinjection procedure at 25 days while anaesthetised, and have their anaesthesia sensitivity (doses of where they fall asleep and wake up) assessed at a subsequent date. Pain relief is provided during the procedure at 25 days, and the anaesthesia emergence protocol at 60 days involves only non-invasive measures of alertness. Animals are euthanised after this exposure.

In other experiments, animals are exposed to anaesthesia and at a later time euthanised. In some cases, animals are euthanised during the anaesthetic procedure, or during emergence (waking up). The latter is to assess changes in cell and molecular endpoints that occur during emergence from anaesthesia.

For the experiments requiring isolation of biological samples from animals exposed to anaesthetics, the animals are simply exposed to normal anaesthetic agents (isoflurane, sevoflurane) and humanely euthanised. This results in minimal pain, suffering, and distress.

All our animal handling will use low-stress handling methods.

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

Assessing mammalian responses to anaesthesia, such as emergence, and the role of astrocytes, a specialised brain cell type, requires the use of a mammalian model.

Whenever possible, we use primary astrocyte cell cultures, but studying emergence ('waking up') from anaesthesia requires an intact and mature organism. The processes involved in the outcomes studied are unique to mammals (not present in organisms such as nematodes, flies, or fish), as are some of the brain structures and functions implicated in the anaesthetic responses we are investigating. Immature life stages (pre-natal, neonatal) cannot be used as anaesthetic responses are quite different in immature animals.

How will you refine the procedures you're using to minimise the welfare costs



(harms) for the animals?

The staff involved in the surgical techniques will have taken hands-on training at the internationally recognised mouse breeding and research centres in stereotactic surgery/injection and perform extensive practice under supervision of experienced staff. Procedures will include proper analgesia and score sheets developed in collaboration with the veterinary staff will be used to monitor animal stress and health. Refresher courses and practice will occur prior to initiating those experiments; this will include practice on a number of carcasses. Outcomes will frequently be discussed with the veterinary and technical staff and refinements added as deemed appropriate.

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

Alongside the guidelines listed below, I will also adhere to local AWERB standards for research animals, and, where appropriate, support the development of new local standards for refinements discovered during the project licence. Where these guidelines conflict, we will select the most refined option.

Code of Practice for Housing and Care of Animals Bred, Supplied or used for scientific purposes.

LASA Guidelines

RSPCA Animals in Science guidelines

UFAW Guidelines and Publications

NC3R's and Procedures with Care

I will consult with the Colony Manager, veterinary staff, and technical staff to review genetic health, best breeding practices, and overall colony health and management at regular intervals. I will consult with animal facility technical staff and veterinary staff on a regular basis and seek out refinements through these interactions. We will involve animal technicians and veterinary staff in assessment of the surgical skills of our staff members performing procedures and the outcomes of procedure practice. If ever needed, we will use the fee-based services of skilled animal facility staff to perform procedures for us.

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

The local AWERB (Animal Welfare Ethical Review Body), NIO (Named Information Officer), NAWCO (Named Animal Care and Welfare Officer), NTCO (Named Training and Competency Officer) and Veterinary team regularly inform, and disseminate, improvements and recent studies involving reduction, replacement and refinement alongside external resources including (but not limited to); collaborators, peers, conferences and lab animal and animal welfare bodies.

During the 1, 3, and 5-year review of the project licence I will update implementation and consideration of the 3Rs that has occurred during the previous period, alongside a review of the linked training plan, score sheets etc. in collaboration with the NACWO, NTCO, NIO and Veterinary team with a particular focus on refinements.

In addition, I will regularly attend national and international conferences in my field, taking note of any advances in methodology that could be used to refine the work in this project, any advances in techniques that may lower the number of animals needed, and any new



models which may be used to address any of the scientific questions raised in this project. Our laboratory is actively involved in developing additional models of disease, which may in some specific areas allow for further reduction in animal use.

A retrospective assessment of refinement will be due by 16 November 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?



12. Treatment and Prevention of Inflammatory Diseases

Project duration

5 years 0 months

Project purpose

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

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

Arthritis, Colitis, Dermatitis

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

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 develop novel therapies to treat inflammatory diseases.

A retrospective assessment of these aims will be due by 14 November 2029



The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

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 diseases affect millions of people worldwide causing pain, impaired function and diminished quality of life. By contributing to the development of new anti-inflammatory drugs, our project will benefit the patients, improving their quality of life, help maintain their independence and reducing suffering. By providing high quality services and scientific expertise, we can make the testing of such drugs more cost effective, more informative and reduce the need for companies to set up the models in house.

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

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

New information on beneficial effects when compared to a vehicle.
New information on beneficial effects when compared to a reference drug.

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

The data generated will typically be sufficient for the study sponsor to decide the lead compound's future: (i) pre-clinical toxicology, the next step in the drug discovery process leading to a novel therapy for an autoimmune or inflammatory disease, (ii) additional efficacy studies or (iii) rejection for lack of safety or efficacy.

Initial assessment in vivo may help to determine tolerability bioavailability and stability of potential treatments to help determine appropriate dosing regimen.

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



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

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

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

By contributing to the development of new candidate drugs for the treatment of autoimmune diseases and inflammatory diseases, we can help expediate the progression of novel compounds by several (2- 5) years from the laboratory to the clinic. As a consequence, patients will benefit from improved treatments reducing pain, swelling and organ damage associated with such conditions, leading to healthier, more independent lives. Typically, we test approximately 100 compounds a year in inflammatory/autoimmune disease models.

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

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

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

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

Species and numbers of animals expected to be used.

- Mice: 8,000
- Rats: 1,500

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.

Many different strains of both rats and mice have been established which provide models of a range of human diseases. Due to their size, ease of reproduction and handling, many aspects of the immune response, leading to disease caused by inflammation and autoimmunity, have been well characterized. The genetics of these strains is well understood, aiding our understanding of disease development and potential interventions. Thus, mice and rats, are, for many diseases, the most appropriate species for early assessment of potential novel therapies.



Adult animals are generally the most appropriate to use, however, occasionally we may need to use neonates, newborns, juveniles or aged animals to better assess the impact of a treatment prior to, or at time of disease onset, particularly where development of disease is spontaneous and may occur early or later in life. Further, the immune response can be affected by age, so use of aged animals in some models may also be required.

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

Animals used in this project typically either develop aspects of the disease of interest spontaneously or following disease induction by administration of a disease inducing chemical or antigen associated with autoimmunity.

Treatments will be administered either prophylactically or therapeutically by routes which in themselves will cause only minor transient discomfort and will involve anaesthesia, with recovery, as required. In some cases, the chemical may cause some mild, transient adverse effects including bodyweight loss. Bodyweight loss will be monitored, and additional nutrients supplied in the form of gel or mashed food on the cage floor. Antigens may be administered with adjuvants such as complete Freund's adjuvant which can cause some localized swelling, this will be monitored, and advice sought from our dedicated veterinary surgeon if a wound develops.

Animals may be bled, in-life, for assessment of immune response, involving restraint for a short time, rats will be acclimatized to restrainers in advance to minimise the potential stress involved. Where test articles are suitably labelled animals may be imaged for assessment of biodistribution under anaesthesia, some disease parameters may also be assessed by imaging. Animals will generally be killed humanely at the end of the study, or following terminal anaesthesia to allow for perfusion or collection of terminal blood.

Duration of studies vary but typically last 4 to 6 weeks.

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

When inducing inflammatory diseases, we expect to see some clinical signs relating to the disease. When inducing arthritis in mice and rats under our treatment and prevention of joint inflammation protocol, this is likely to cause adverse effects such as joint swelling and reduced mobility. For our treatment and prevention of gastro-intestinal inflammation projects the adverse effects we expect to see are bodyweight loss, diarrhoea, intestinal bleeding and abdominal discomfort. On our treatment and prevention of skin inflammation projects we expect to see changes to the skin such as skin thickening, flaking, crusting and redness. Adverse effects expected on the treatment and prevention of peritoneal inflammation project include body weight loss and changes to appearance such as coat condition, posture and lethargy. Our air pouch model is not expected to cause adverse effects.

The expected level of severity for all the above models is moderate. Measures are taken to limit harms such as frequent monitoring of disease-specific clinical signs and non-specific clinical signs for early identification of adverse events. Moderate signs are not tolerated for more than 24 hours and severe signs will not be tolerated. At the end of an experiment, all animals will be humanely killed to enable further in vitro testing of samples.

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 most protocols involve the development of clinical signs associated with disease, the majority of mice and rats, approximately 90%, are expected to experience moderate severity.

A few naïve animals, <10%, may be included to provide baseline levels of disease, cytokines, cell proportions or numbers, for comparison with treated and untreated controls.

What will happen to animals at the end of this project?

- Killed

A retrospective assessment of these predicted harms will be due by 14 November 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

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?

Inflammation, the immune system's response to the presence of antigens, involves multiple systems, multiple organs and multiple cell types. The complexity of the inflammatory response cannot be reproduced in laboratory tests alone.

In addition, the symptoms of inflammation - heat, redness, swelling and pain- cannot be modelled in a laboratory. Experiments on cell lines and on cell cultures will be performed to help understand the potential of a compound to interact with immune cells. However, the limitations of these methods do not allow them to replace the use of experimental animals: there is no alternative to the use of a living animal that would allow the objectives to be met and progress potential therapeutics to the clinic.

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

Clients will often provide data from assays already performed that indicate the potential of a compound to reduce the onset of inflammation or autoimmune disease.

In the absence of such data, in vitro assessment may be required before progressing to an animal model. We have a number of in vitro cell based assays including use of transwells and 2D models. We are also developing the use of organoids and tissue on a chip models for assessment of test compounds prior to testing in animals. In addition, the wax moth larvae, *Galleria melanogaster* model, may also be used as an intermediate between in



vitro assays and rodent models, as this has a basic innate response, enabling some aspects of cellular interactions to be assessed in context of the therapeutic of interest.

Why were they not suitable?

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

A retrospective assessment of replacement will be due by 14 November 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your experience?

Reduction

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

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

This is estimated from previous animal returns.

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

Use of in vitro assays, including potential 2 and 3-dimensional set-ups, will minimise the number of animals required for initial assessment of potential novel compounds. In addition, utilising the National centre for implementation of replacement, reduction and refinement of animal use in research (NC3R's) guidelines and the experimental design assistant, can help to balance group sizes. Numbers of naïve, untreated or vehicle treated animals will be kept to a minimum while still allowing meaningful data to be assessed for statistical significance. Objective of the study (e.g., a dose finding or efficacy) as well as the incidence of disease and variability of the model will impact group sizes. Assessment of previous data or review of the literature will also help guide the group sizes as well as the number of control groups required. While we work to Good Laboratory Practice (GLP)-like standards, however, we do not have to comply to additional regulatory guidelines.

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

Understanding from the literature and breeding establishments the expected incidence of disease and variability of the model will help ensure group numbers are appropriate. Assessment of previous data or review of the literature will also help guide the group sizes



as well as ensuring appropriate control groups included.

A retrospective assessment of reduction will be due by 14 November 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

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.

Appropriate models are assessed by review of the literature to ensure the most up to date refined model is used. Reducing where possible the number of administrations required, minimising administration volumes and sampling frequency and volume. We will use the shortest model that will provide us with suitable disease profile and incidence of disease for assessment of treatments.

Use of specific strains or GA mice such as the NC/Nga mice can provide a more relevant model of disease for assessment of potential therapies such models will therefore be used where appropriate.

Animals are housed in groups and kept in an appropriate environment with plentiful bedding and nesting material and suitable object that allow them to express normal behaviour. All staff are trained in good animal handling procedures. Animals are always handled gently and humanely, especially animals which may be in pain. Animals may be acclimatised to being handled prior to the experiment starting so that they are less stressed once the study begins.

Animals are provided with a bowl of mashed food on the cage floor if moving may be uncomfortable. When substances need to be administered, we will give the smallest volume possible and administer it in the way that causes the least distress.

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

Most of our studies will be done in mice, which are the lowest species that develop these diseases in the same way as humans. Rats are occasionally used when the disease cannot be modelled in mice, if the test compound does not work appropriately in mice, or if a larger animal is needed for sampling requirements.

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



Animal suffering will be limited by ensuring that the models used cause the least amount of harm to the animals. The mildest disease inducing agent or dose will be used, and studies will be kept as short as possible. Animals are monitored frequently for signs of discomfort, and appropriate action taken promptly. We will monitor animals closely throughout the studies, and they will be treated or humanely killed if they develop signs of excessive suffering.

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

We follow the recommended Best Practice guidelines of Turner et al. 2011 and Diehl 2001.

We keep up to date by receiving regular updates from the Home Office (HO), Laboratory Animal Science Association (LASA), as well as the IAT, following the literature, internal forums and advice from our dedicated veterinary surgeon.

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

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

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

A retrospective assessment of refinement will be due by 14 November 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?



13. Evaluating medical countermeasures for dangerous pathogens

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

Infection, Medical countermeasures, Bacteria, Virus, Therapies

Animal types	Life stages
Mice	adult
Rats	adult
Hamsters (Syrian) (<i>Mesocricetus auratus</i>)	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

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

What's the aim of this project?

The overall aim of this project is to develop appropriate animal models of infection caused by a variety of dangerous pathogens, and to use these models to assess protection of therapies (Medical Countermeasures (MedCMs)) to combat these diseases in humans. Examples of therapeutic MedCMs include antimicrobial drugs, immunomodulatory drugs,



vaccines and/or antibodies that may be administered individually, in combination or in the context of nanoparticle delivery systems.

A retrospective assessment of these aims will be due by 24 November 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

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 licensed therapies available to treat or prevent infections caused by the dangerous pathogens to be studied, and many of these are limited in their efficacy, particularly against respiratory infection. Therefore, there is a need to devise and refine therapies that can be used to treat these infections in the event of an outbreak in human populations.

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

The overall benefit of this work is to identify potential therapies that will provide high levels of protection against specific dangerous pathogens. Promising therapies identified on this project may be further evaluated in a second animal species such as non-human primates (as appropriate) on other Project licences, to support advanced development of candidate medical countermeasures, which may ultimately lead to licensure. Benefits of work on this Project licence include new information to:

- 1) Increase our understanding of the infectious process of these pathogens, including the effect of inhalational infection where a greater understanding of host-pathogens interactions including the identification of biomarkers, and will enable identification of opportunities for therapeutic intervention.
- 2) Understand the pharmacokinetics of compounds to allow the selection of an appropriate dose to be used in our models, and if known, select doses that are comparable to doses used in humans.
- 3) Understanding how much available therapy there is following a dose or the scale of an immune response within e.g. the blood or other body tissues, will also help to inform the selection of appropriate doses or concentrations that are likely to provide a protective effect.



- 4) Identify suitable candidates for future development for use in humans.

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

In the short term this information will inform the scientific research community on which animal models are appropriate to assess efficacy of therapies for a variety of dangerous pathogens. This will help inform the direction of future research. In the longer term therapies developed as part of this project can be used to protect the UK against infections caused by dangerous pathogens for example in the event of an outbreak. The benefit of this project also extends globally, where therapies could also be exploited for humanitarian benefit in regions where these diseases are endemic.

Additionally, this work supports the UK's Biological Security Strategy with the mission to implement a UK-wide approach to biosecurity which strengthens deterrence and resilience, projects global leadership, and exploits opportunities for UK prosperity and science and technology (S&T) advantage.

(<https://www.gov.uk/government/publications/uk-biological-security-strategy/uk-biological-security-strategy-html>).

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

Data generated from these studies will be shared via open access publication and at scientific forums. International linkages also allows exploitation of data, collaboration and burden sharing. Data may also be used to develop in silico models or alternative models to further our understanding of these models without the further use of animals.

Species and numbers of animals expected to be used

- Mice: 7075
- Rats: 424
- Hamsters (Syrian) (*Mesocricetus auratus*): 930

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 to evaluate new or existing therapies to improve treatment of dangerous pathogens, this may include vaccines to protect you from becoming ill if you are exposed to a dangerous pathogen, therapies that can treat you if you think you may have been exposed or treatment therapies for when you have become ill from a dangerous pathogen. In the first instance, laboratory studies will be completed to determine if the drugs are efficacious against the microorganism of interest outside of an animal model. However ultimately a complete physiological system with a range of interacting body systems



(immunological, respiratory, endocrine etc) is required to determine whether they are efficacious in human disease. Therefore, we will use the lowest order animal that exhibits representative disease which will be mice, rats or hamsters (depending on the pathogen).

In all cases adult animals will be used, this is because a fully developed immune system is required to evaluate these therapies against dangerous pathogens. The type of animal used will depend on the pathogen being investigated and the most appropriate model will be selected, for example mice do not get infected with Nipah virus, however other rodents do and the disease is similar to that seen in humans, therefore hamsters will be used as they are susceptible to this pathogen and are the next appropriate model if a mouse can not be used.

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

1. Most animals will be used to assess how well vaccines or medicines or a combination of these can protect against or treat disease. For these studies, the general experimental approach will involve:

- On a single occasion animals will be exposed to pathogen. This will usually be delivered as an aerosol, by the inhalational route which would involve physical restraint, where the animal is placed in a tube that secures its body and its nose protrudes from the end of the tube. The duration is typically 20 minutes but could be up to a maximum of 30 minutes. Sometimes the pathogen will be given to the animals by injection (e.g. subcutaneous, intraperitoneal or intravenous) or by the oral or nasal route.
- Before or after challenge depending on the nature of the medicine, animals will be given a vaccine or medicine (or a control substance) or combinations of medicines and vaccines to assess whether this prevents or treats disease, or offers a better outcome compared to the currently used medicines or vaccines. A vaccine may be given up to 5 times by injection using standard routes (e.g. subcutaneous). The medicines may be given up to three times a day for up to 21 days, most commonly this will be by the oral route but some animals may receive the medicine by injection or inhalation, if it was by inhalation this would only be given once a day. Some animals may receive a combination of medicines or a combination of medicines and vaccines.
- In some studies animals may be sensitised to pathogen (nasal route) or have non-pathogenic adenovirus administered by injection or the nasal route on one occasion. The purpose of this is to make animals susceptible to a disease that they would not normally be susceptible to.
- Blood samples may be collected before challenge. Blood samples may be collected at the end of the study and this would be performed under terminal anaesthesia.



- On some occasions an immunomodulatory substance may be used to determine if relapse is seen, this is typically administered by injection once a day for up to 5 days.
- In a small number of experiments, animals may be imaged which involves an injectable anesthetic and then placement in a scanner.

A typical efficacy study would last 4 weeks, involving 2 weeks of treatment followed by 2 weeks of observation, however for some studies particularly if a vaccine is to be evaluated studies could last up to 22 weeks.

2. Some animals will be used in studies aiming to understand how vaccines or medicines are processed in the animal (e.g. the immune response to a vaccine or how quickly the drug is expelled or degraded) to ensure the treatment regimens used are similar to those used in humans. These studies may be for medicines or combinations of medicines, these are typically short in duration as medicines are processed quickly in animals, typically within 24 hours. Or the studies could be for vaccines or a combination of medicines and vaccines where the studies are typically 10 weeks as time is needed for the animal to produce an immune response. The general experimental approach will involve:

- For medicines, typically the medicine or combination of medicines will be given on one occasion, most commonly this will be by the oral route but some animals may receive the medicine by injection or inhaled. However, on some studies the medicines may be given up to three times a day for up to 7 days, These studies are typically very short, less than 24 hours but some may last up to 7 days.
- For vaccines. A vaccine may be given up to 5 times by injection using standard routes (e.g. subcutaneous).
- Blood samples may be collected after vaccination, this would typically be on one occasion via the tail vein. Blood samples may be collected at the end of the study and this would be performed under terminal anesthesia.

These studies are typically 10 weeks long to allow for an immune response to be produced.

3. A small number of studies will look at how a pathogen causes disease in an animal in the absence of therapeutic intervention. The aim of this is to develop models that can be used to study treatment and ensure the models are suitable. For these studies, the general experimental approach will involve:

- On a single occasion animals will be exposed to pathogen. This will usually be delivered as an aerosol, which would involve physical restraint, where the animal is placed in a tube that secures its body and its nose protrudes from the end of the tube. The duration is typically 20 minutes but could be up to a maximum of 30 minutes. Sometimes the pathogen will be given to the animals by injection (e.g. sub-cutaneous, intraperitoneal or intravenous) or by the oral or nasal route.



- Blood samples may be collected at the end of the study and this would be performed under terminal anaesthesia.

These studies would typically last up to 2 weeks but may last up to 5 weeks, depending on the pathogen being investigated.

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

The most significant impact that most animals may experience is that they will feel unwell from the disease caused by the pathogen. During this time they may not move around as much as normal and not groom as much as usual and may lose weight. They may breathe more quickly or their breathing could become labored. Some pathogens can infect the brain resulting in neurological signs, however, animals are euthanised quickly if this occurs. In animals that do not receive a therapy or where the therapy is not effective the clinical signs of these animals will increase in severity, which would ultimately lead to death if a humane endpoint were not applied. Typically animals will have clinical signs due to disease for 3 to 4 days, but in some cases this can be longer.

Vaccines and medicines given to animals, will have already been in vivo and should therefore have no adverse effects.

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

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

For mice the expected severities are: mild 20%, moderate 50%, severe 30%.

For rats the expected severities are: mild 20%, moderate 50%, severe 30%

For hamsters the expected severities are: mild 20%, moderate 40%, severe 40%

What will happen to animals at the end of this project?

- Killed

A retrospective assessment of these predicted harms will be due by 24 November 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?



Replacement

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

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

A wealth of data can be generated from human clinical trials to provide safety data when developing new medicines, however current legislation dictates that all therapies must be tested in animals prior to first time in human studies. It is not possible to conduct protection studies in humans to protect against the inhaled route of infection as this is not generally a natural route of infection for the pathogens listed on this licence, and it would be unethical to conduct infection trials in humans. It is also difficult to conduct efficacy studies in human populations where these diseases are not endemic. Animal models can be used to predict the efficacy of therapies as they are evaluated in a living system and this data can be extrapolated to human data. A mammalian model will ultimately be required to achieve the aims and objectives of this licence.

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

In vitro systems such as tissue culture assays, ex vivo assays including organ-on-a-chip technology and in silico modelling have been developed to replace the use of protected animals in our research and are utilised as far as possible. In addition, alternative systems using non-mammalian models such as *Caenorhabditis elegans* and *Galleria mellonella* are also being developed and utilised. Data provided by these alternative systems are being used to screen and select the most promising candidates for in vivo assessment on this project licence. We have used hollow fibre assays and modelling to predict the efficacy of antibiotics, reducing the number of animals required for determining drug concentrations in plasma, with further development the use of this may replace the need for animals. We are developing organ on a chip methods to investigate replacing the use of animals.

Why were they not suitable?

Whilst they are able to support the aims of this licence, these systems are unable to provide a fully functioning physiological system to predict the complex interactions between a human host and a pathogen.

A retrospective assessment of replacement will be due by 24 November 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your experience?

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 that may be used has been based on customer requirements and we expect to do 20 mouse studies per year, 3 rat studies per year and 7 hamster studies per year. The numbers are based on what studies we have confirmed over the next 2 years and based on the type of study that we will be performing and we have extrapolated for the remaining 3 years where we expect the requirement to be similar.

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

We have in-house experts who are consulted for statistical advice for each animal study to ensure the number of animals used per study will be the minimum required to provide statistically significant data and to allow the objectives of the experiment to be achieved. Generally, the statistical power will be set to 80% against the desired effect. Experimental design will vary greatly depending on the purpose of each individual experiment and tools such as the Experimental Design Assistant (EDA) provided by the NC3Rs will be used to ensure experiments are appropriately designed.

We have links with academia, national and international organisations where we align our areas of work to ensure that studies are not duplicated.

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

The maximum amount of relevant information will be obtained from each animal to reduce the necessity of repeat studies and increase statistical power. Where appropriate, inbred strains of animals will be utilised in order to reduce the inter-animal variability and reduce group sizes.

To minimise bias, animals will be randomised into cages on arrival and the cages will be randomised into treatment groups where practical. As far as possible, staff will be 'blinded' to treatment groups. When possible, control animals will be shared within a study e.g. when multiple therapies are being assessed in the same study and tissues will be shared across multiple projects. Data generated from animal studies will be shared via publication in the open literature, adhering to ARRIVE guidelines, and at appropriate scientific forums to reduce duplication of experiments.

A retrospective assessment of reduction will be due by 24 November 2029



The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

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 lowest mammalian species will be used that are representative of human infection and are expected to achieve the experimental objectives. In the first instance this will be mice, however, on those occasions where mice are inappropriate for example because they are more sensitive to infection than humans or are not susceptible to infection, alternative species such as rats or hamsters will be investigated. These animal models will be selected to replicate salient features of human infection, especially the severity of infection, infectious dose and the course of disease progression over time.

Rats have been chosen for work involving vaccines and *Francisella. tularensis* because it is known that this species responds in a more human-like manner to the Live Vaccine Strain of *F. tularensis* than does the mouse and the expectation is that it will respond in a similar fashion to experimental vaccine candidates. Mice do not appear to be susceptible to Nipah virus by standard route of infection (e.g. aerosol, intra-peritoneal) and as such cannot be used to model disease. Alternative species which have been reported in the literature include cats, pigs, fruit bats, ferrets, hamsters and African green monkeys. Of these, hamsters appear to mimic human disease most closely and are perceived to have a low neurophysiological response and are therefore the most refined choice of animal model.

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

We can't use embryos or very young animals as their immune system is immature and doesn't respond to pathogen in the way mature animals do, and therefore can not be used to evaluate efficacy of medical countermeasures.

The time to onset of disease and the effect of the therapies occurs over many days and weeks and it would not be possible to do this in terminally anaesthetised animals. In addition the assessment of clinical signs is dependent on observing behaviour in a conscious animal.



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

Methods for delivery of pathogen or a therapy will be refined as much as possible e.g. oral delivery via pipette instead of oral gavage. Where appropriate, animals will be familiarised to the procedure (e.g. handling, environment). For inhalational exposures, animals will be restrained for the minimum period (usually ~ 20 minutes but up to a maximum of 1 hour). Animals will not be acclimatised to the exposure system as this would likely result in a cumulative harm to the animal that would markedly exceed the harm from restraint required for exposures in these studies. Methods to increase the efficiency of delivery of pathogen or therapies will be investigated to reduce the overall restraint period. Animals may be briefly anaesthetised using inhaled agents such as isoflurane e.g. for oral gavage, intranasal instillation or for placement within restraint tubes.

Animals will be monitored throughout the infection and when a peak in development of clinical signs and possible mortality is expected, the number of checks will be increased to a minimum of 4 hourly to minimise the number of animals experiencing severe disease.

We have refined humane endpoints for some diseases where we have enough data, resulting in animals experiencing less suffering but still achieving the scientific outcome. We will continue to refine humane endpoints as much as possible.

We will trial the use of new cages that provide real-time monitoring of digital biomarkers (temperature and activity) to maximise the data output and assess its use in detection of humane end points.

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

We will follow the PREPARE guidelines when planning studies. The NC3Rs website will be monitored for new guidance and publications and we will refer to these and use the best practice for the administration of substances and removal of blood, including routes and volumes' for appropriate dosing regimens (for example Turner et al (2011)).

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

We promote and support activities that enable those working with animals to remain informed about advances in the 3Rs and incorporate these effectively across projects. This includes information distributed by the NIO, the provision of internal funding to support technology watch activities, purchase new equipment and attend relevant conferences/meetings. There is also a strong culture of sharing of information within the project team and also across the organisation which is provided through various forums/committee meetings.



For every study throughout the 5 year lifecycle of the licence we will review the 3Rs and look for opportunities to introduce additional interventions where appropriate.

A retrospective assessment of refinement will be due by 24 November 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?



14. Immune responses important for controlling infections and cancer progression in the lung

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

Infections, Cancer, Lung

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

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

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 how respiratory infections cause severe disease and how the cross-talk between lung cancers and respiratory infections dictate disease outcomes.

A retrospective assessment of these aims will be due by 29 November 2029

The PPL holder will be required to disclose:



- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

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 lung is a vital organ for our survival and therefore lung infections and lung cancers are both important causes of morbidity and mortality. As evident during the COVID-19 pandemic, infectious pathogens can cause considerable global disruption, disease and death. Respiratory infections with viruses, bacteria and fungus are leading causes of hospitalisation of infants and they frequently cause life-threatening illness in elderly and immunocompromised people. Respiratory pathogens can cause severe disease in the lung which is not always due to a massive proliferation of the pathogen but rather due to an over-active or dysregulated immune response to the infection. In order to improve treatment and avoid severe disease we need to elucidate the mechanisms of how pathogens cause disease and what role the immune system plays in this.

Furthermore, lung cancers or metastatic tumour cell spread to the lungs are huge health burdens. Lung cancers or lung metastatic cancers are common and very lethal cancers. Lung metastatic cancers are cancers that form when cancer cells break away from another tumour, travel through the blood or the lymph and then form new tumours in the lungs. There are around 35,000 lung cancer deaths in the UK every year, accounting for approximately 25 percent of all cancer deaths. The interplay between cancers and lung infections is not fully understood and a more mechanistic understanding of how the immune responses in the lungs influence infections and cancer is critically needed to develop novel therapeutics and treatments. We will study both primary and secondary lung cancers within the same programme of work and are therefore likely to provide added benefits through direct scientific comparisons of disease outcomes and the mechanisms of protection against cancer development and growth.

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

We aim to understand how respiratory infections cause severe disease and how the cross-talk between lung cancers and respiratory infections dictate disease outcomes

This work will:

- generate a detailed dissection of the immune response to respiratory infections. This will increase the knowledge of how the immune system senses the pathogens to the complex interactions between the different cell types that form the immune response leading to both protection and in some instances increased disease severity.



- enhance our knowledge of how disease following infections or co-infections develops and will potentially identify targets for future interventions.
- dissect the underlying mechanisms of how lung infections influence the initiation and growth of lung cancers and the seeding and growth of metastatic cancer cells.

Outputs of this work will be disseminated to the scientific community through publication in peer-reviewed, open-access journals and presentation at scientific conferences. We will also make new transgenic animals available and share tissues collected at the end of experiments whenever possible. In addition, we will collaborate with clinicians and drug companies to test possible novel therapeutics.

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

In the short/medium term:

Knowledge generation: the data from this project will contribute to the scientific understanding of lung infections and cancer. This knowledge and outputs will benefit other scientific researchers, the pharmaceutical industry and healthcare professionals around the world.

In longer term:

The knowledge generated will contribute to a broader understanding of the immune response to infections and cancer. These questions are important to other immunologists and cancer biologists. The work, in the long run, will also benefit clinicians and patients.

Product development and validation: Our models will be of possible interest to industry partners for testing of novel therapeutics or vaccines.

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

1. Collaboration with academic and industrial partners.
2. Dissemination of knowledge through standard scientific routes (papers/ meetings), social media (blogs/Twitter (X)) and scientific engagement (festivals, working in schools etc).
3. Where appropriate, unsuccessful approaches and negative data will be published as part of larger data sets.

Species and numbers of animals expected to be used

- Mice: 12520

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 only using mice in these studies. Mice recapitulate many aspects of the human immune response. Mice and humans have about 30,000 genes of which 1% are species-specific. Equivalent mouse genes have been found for all genes known to cause human disease, and 99% of mouse genes have a human homologue.

There are many benefits to the mouse model: the mouse is the only species for which many of the research tools are available to study mechanisms of immune responses. The mouse genome is one of the best characterised, with many knockout and transgenic strains (mice that lack certain genes or genes that have been replaced with for example human genes or inserts that make it possible to trace certain cells) available to investigate important research questions. Other species may be moderately more reflective of some aspects of the human immune response, but the interventional mechanistic studies proposed are simply not possible in them. All studies will be informed by in vitro work to determine the correct doses of virus and drugs. For example, doses of the viruses and toxic effects on cells will be tested in vitro on cell lines. We will also use cell lines and purified lung cells for in vitro/ex vivo studies of functions of specific cells. However, in order to understand the complete immune responses these studies will inform or be performed in parallel with the in vivo studies explained in this licence.

We will also, in some studies, be using neonatal, juvenile or elderly mice. In general, respiratory infections are more severe in early or late life. Some of this is driven by an immature or declining immune system. Performing comparisons between early and later life is important to understand these differences.

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

We are interested in the how the immune responses to infection, predominantly respiratory infection (e.g. RSV, influenza virus and SARS-CoV-2) are initiated and regulated to protect, and in some circumstances cause more severe disease.

Most animals will receive some form of infection. This will normally be in the context of immune modulation using for example knockout mice or immune modulatory therapies. Most animals will undergo a single infection but when studying memory responses several infections might be necessary.

Standard experiment:

Animals will undergo the following typical procedures:

A. To investigate disease after a respiratory infection



- 1) Use of mice that are genetically altered for specific genes of interest or drugs to manipulate disease severity; these drugs will either be administered intranasally (the site of infection) or systemically by injection.
 - 2) Some mice will go through irradiation in order to reconstitute their bone marrow compartment so that immune effects can be studied in different cellular compartments (bone-marrow derived cells or stromal cells).
 - 3) Some mice will be treated with immunomodulating agents such as vaccination, antibodies or cytokines. These agents will be administered via different routes (intranasally, orally, systemically, intraperitoneally (injection into the body cavity).
 - 5) Pathogen or inflammation-induced agent inoculation via the intranasal route, under general anaesthetic; normally a single infection, but occasionally we will assess the effect of one infection upon another.
 - 6) Monitoring infection severity through signs of disease in infected animals using for example weight loss.
 - 7) From some mice, blood samples might be taken.
 - 8) Mice will be humanely killed. A normal infection study will range from very acute (1-4 days post infection) to longer time (1-3 weeks post infection) to allow the immune response to develop.
- B. To investigate how respiratory infections influence lung cancer or metastatic cancer cell spread
- 1) Mice get a carcinogen administered intraperitoneally (injection into the body cavity) to initiate lung cancer or they get an intravenous injection (injection directly in the blood stream) with metastatic cells that will spread to the lungs. These mice could be genetically altered mice deficient in specific immune pathways.
 - 2) Pathogen inoculation via intranasal inoculation, under general anaesthetic; normally a single infection.
 - 3) Mice will be humanely killed. The carcinogen studies last up to 3 months to allow for tumours to develop.

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

The procedure that is most likely to cause adverse effects in the animals is infection.

Infections will occasionally result in some transient discomfort for the animals. This is most likely to be seen in control-unimmunised or untreated mice. But the use of infection is



central to these studies and as we are studying lower respiratory tract infections some signs of disease is unavoidable to address our research questions.

Most infections will lead to transient adverse effects. These include clinical signs such as lethargy, faster breathing and a small amount of weight loss.

Occasionally, mice will experience more severe, transient responses to infection, including substantial loss of body weight (up to 25%) and sustained signs of ill health (such as greatly reduced activity and rapid breathing) over a period of several days. The duration depends on the infecting pathogen, but there is normally a 3-day period over which the adverse effects peak before recovery or humane killing.

Some other procedures – for example drug administration, vaccination, chemotherapeutics or irradiation can cause a low level, short term weight loss and reduced activity for less than 72 hours.

In the different cancer models; chemically-induced lung cancer, metastatic lung cancer or subcutaneous/mammary fat-pad induced breast cancer will rarely give rise to any general signs of ill health as the experiments will stop before the tumours reach a size that can cause any clinical signs.

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

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

Sub-threshold 28%

Mild 18%

Moderate (infection or immunomodulation studies) 43%

Severe (minority of infection studies) 11%

What will happen to animals at the end of this project?

- Killed
- Kept alive
- Used in other projects

A retrospective assessment of these predicted harms will be due by 29 November 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?



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 based on the study of the interactions of pathogens, cancer and the host in the context of an intact immune system. Investigating individual cells is informative and in vitro studies are being performed in parallel. However, the immune response comprises multiple different cell types working in concert and some of the analysis of this multi-component system can only be performed in vivo using animal models.

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

A limited amount of pilot data can be obtained from in vitro work and ex vivo work using cell lines and tissue. We have started to use precision cut lung slices (from tissue both from mouse and human), which we can expose ex vivo to viruses or compounds and test how resident lung cells respond.

We will test novel viruses and chemicals on cells from cell lines to estimate virulence and cytotoxicity. We will also use lung tissue from humans to validate some of our findings.

In addition, we also work collaboratively with a group performing human virus challenge experiments. Via this collaboration we can benefit from human data that can advise and guide our experiments.

Why were they not suitable?

In vitro cell models and ex vivo tissue models can only inform us about how individual cells respond to the infections, but not about the complex interplay across multiple cell types. One question of particular importance is the kinetic of the response between injection site, lymph node (where some of the immune responses are initiated) and the potential infection site and how these sites work together. In vitro models cannot give us this information.

The human viral challenge studies cannot replace the animal models as they are limited in the disease severity and also in mechanistic insight that you can obtain, especially from the lower airways.

In addition, the cancer models include growth of tumours in the microenvironment of the lungs with infections at different times of tumour development. This is impossible to recapitulate in vitro or ex vivo as an intact animal is important to study the immune response.

A retrospective assessment of replacement will be due by 29 November 2029



The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your experience?

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 draws on previous experience/ earlier project licenses and the volume of work required to confidently address the scientific questions.

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

The studies are carefully planned to reduce animal numbers, overlapping control groups are used where possible.

We will only perform studies on animals when there is no other alternative. We will reduce the numbers of animals used by extensively testing our hypothesis in experiments without animals before confirmation studies in animals.

When it comes to the use of animals, we will use statistical advice and our longstanding experience to minimise the number of animals needed to answer each research question. Experimental design is informed by ARRIVE guidelines.

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

We will continue to use efficient breeding strategies and manage colonies and litter size to breed enough to be used in experiments but not in excess.

We will start all our experiments with a small pilot experiment with of a few animals to be sure that there will be no unexpected welfare issues and to see if it is justified 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, to perform experiments in the laboratory so no additional animals are required.



The use of sufficient pathogen to result in transient weight loss provides a method for non-invasive monitoring of disease meaning effects of treatment can be rapidly evaluated without the use of larger numbers of animals.

A retrospective assessment of reduction will be due by 29 November 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

Refinement

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

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

Mice have been chosen for this study for several reasons, there is a large number of tools available to interrogate the immune response, they are widely used so responses are comparable between different studies and they are the lowest mammal in which these studies can be performed and they recapitulate most features of the human immune response.

The most severe procedure in the studies is the use of infection (e.g. influenza virus, RSV, SARS-CoV- 2). The central goal of this project is to understand what causes disease. We are mainly using respiratory infection models (mostly viral). We have more than 25 years' experience working with these models and have refined them throughout this time. We use the lowest dose to cause symptomatic disease, where new batches of pathogen are used, we perform small pilot studies to ensure the dose causes the minimum harm. Animals are closely monitored to ensure harms are minimal. Additional support, for example food on floor of cages, extra bedding and occasionally wet mash are used.

Studies are terminated in accordance with severity and scientific endpoints, to ensure each study generates high quality data that will address the aims of the project. Most infection studies are terminal, or lower doses are used where recovery is needed to look at long term immune memory.

The cancer models that will be used are closely resembling human cancer and are also well defined tumour models. We will monitor the tumour bearing mice closely so that no



animals suffer due to the tumours. We will also use the shortest duration possible for the experiments.

Other procedures are mild or cause transient low level distress in the animals. These all draw on previous experience and are refined to cause the minimum suffering, for example through anaesthesia where pain might be local and transient.

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

The immune responses to infections are complex, multi-stage responses. We need to investigate changes over time. So terminal anaesthesia is not possible.

Non-mammalian animals are limited in their use because they either do not have the right type of immune cell or their immune system is too different from the human immune system to provide relevant results. We can't use embryos or very young animals as their immune system is immature and doesn't respond to antigenic stimulation in the way mature animals do.

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

We will always strive to use the lowest doses (pathogens, irradiation etc) possible to achieve our research goals. We will also use the less invasive routes when possible. We will use situation specific monitoring, with increased intensity at known times of severity or for new/ pilot studies. Where problems arise, we shall consult the NACWO and veterinary surgeon and offer pain relief, treatment or humanely kill animals as appropriate.

For infection studies we will monitor the mice carefully especially during the peak of infection and increase the bedding, giving wet food etc.

For tumour models we will monitor the mice frequently to detect any signs of unexpected adverse effects due to the cancer burden.

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 closely for adverse effects.

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

We will follow resources available including guidance and publications from the NC3Rs and Laboratory Animal Science Association, the ARRIVE and the PREPARE guidelines etc.



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 our NC3Rs Programme Manager and attend local and regional 3Rs symposia.

Through reading current literature and discussions at conferences.

A retrospective assessment of refinement will be due by 29 November 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?



15. The interaction between brain and blood glucose in health 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

Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Diabetes, Hypoglycaemia, Dementia, Insulin sensitivity, Hypothalamus

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

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

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 blood glucose is controlled by the brain and other organs and how diseases such as diabetes affect this.

We also aim to understand how changes in blood glucose and related factors such as obesity can, in turn, affect the brain.

A retrospective assessment of these aims will be due by 30 November 2029



The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

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

Why is it important to undertake this work?

Diabetes is a growing and major health problem across the World characterised by raised blood glucose levels which can damage organs such as the heart, feet, kidneys and eyes. Blood glucose-lowering treatment can protect against this damage but some of these therapies such as insulin carry a risk of overshooting into low blood glucose (hypoglycaemia). A subset of people with insulin-treated diabetes lose their natural defences against low blood glucose, making it very difficult to find the right balance when managing their diabetes. We think that this happens because of changes in the specialised brain cells which detect hypoglycaemia and, in this project, we aim to identify these changes. If successful, this work may allow new treatments for diabetes to be developed with lower risk of hypoglycaemia.

As well as protecting against hypoglycaemia, the brain is also involved in detecting and responding to high glucose. A second important part of our work is to identify the brain pathways which are active with high blood glucose, again with the ultimate aim of finding targets for new effective and safer blood glucose lowering treatments to be developed for diabetes.

Finally, as well as damaging organs such as the heart, feet, kidneys and eyes, diabetes and related disorders such as obesity increase the risk of dementia. We will examine whether this might be explained by changes in the small blood vessels within the brain, again working with the ultimate aim that new treatments that act on small brain blood vessels could be developed to reduce the risk of developing dementia.

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

Identify at least one brain area containing cells which detect **low blood glucose** and trigger defensive responses. We will examine how this area changes in diabetes using rodent model(s) that mimic human diabetes. We will also examine whether we can target this area (at least under experimental conditions) to boost defences against low blood glucose.

Identify at least one brain area containing cells that detect **high blood glucose** and trigger corrective responses to help restore blood glucose back into the normal range. We will examine how this area changes in rodent model(s) of human diabetes and whether we can target this area to lower blood glucose.

We aim to identify whether diabetes and/or obesity can result in **changes in small blood vessels** in rat or mouse brains associated with a decline in brain performance. If so, we will then examine whether experimental treatments targeting these small blood vessels



can protect the brain against the effects of diabetes/ obesity.

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

Output 1) During the lifetime of this licence, characterising how defences against low blood glucose are lost and whether we can protect/ restore these in rodent models will be useful information for us and other research groups interested in the problem of hypoglycaemia in diabetes. We will share our findings with others in the biomedical scientific community and this may allow others to build on our findings. The work may also be of use to scientists working to understand the basic control mechanisms of the body (called "homeostasis") as maintaining blood glucose is an essential "housekeeping" function of life.

Beyond the scope of this 5-year licence, this rodent work may allow drug treatments to be predicted and tested in future studies in humans with diabetes. This could include new drug treatments or even finding a new purpose for existing treatments (called re-purposing). This is particularly relevant for the 20% of people with type 1 diabetes who have lost their defensive responses and awareness of low blood glucose. In the 2021/2022 National Diabetes audit, there were 260,000 people in England with type 1 diabetes with 20% (50,000) likely to have a problem with noticing and responding to low blood glucose.

Output 2) During the lifetime of this licence, characterising whether brain areas can be targeted to lower blood glucose in rodent models will be useful for us and other research groups interested in finding new treatments for diabetes. As above, we will share our findings with others in the biomedical scientific community including those interested in "homeostasis".

Beyond the scope of this 5-year licence, this work may allow novel blood glucose-lowering drug treatments to be predicted and tested in future studies in humans with diabetes. This is particularly important for people living with type 2 diabetes where even with advances in glucose-lowering therapies, many people still have average blood glucose levels higher than target values. In the 2021/2022 National Diabetes audit, there were 3.3 million people in England with type 2 diabetes with only 30% of these achieving ideal glucose targets leaving 2 million living with chronically high blood glucose values.

Output 3) During the lifetime of this licence, characterising whether small blood vessels in the brain change in rodents with diabetes/ obesity and whether targeting these small blood vessels is protective will be useful for us and other research groups interested in diabetes and dementia.

This may allow drug treatments to be developed and tested in rodents and potentially (beyond the scope of this 5-year licence), in future studies in humans with diabetes and early changes of dementia. As an example, we work with industrial collaborators who have developed drugs which can increase the number of small blood vessels in tissues. This work will be important for the growing numbers of people with diabetes and dementia. In 2023, there were just under 1 million people living in the UK with diagnosed dementia, with the presence of diabetes (around 6% of the overall population) being reported to increase the risk of dementia by 60%.

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

Work will be presented and disseminated to clinical and scientific audiences by



presentation at meetings, and publication in journals including pre-prints.

Increasingly, we use social media to disseminate scientific findings, especially Twitter (X). 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.

There is also increasing focus with colleagues in the Open Science initiative to make data accessible to all.

More broadly, we are focused on allowing the "translation" of pre-clinical science into real advances in healthcare that can impact people's lives. The PPL holder is the nominated translational champion and helped create a strategic translational strategy. 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: 7755
- Rats: 400

Predicted harms

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

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

We will use adult mice and rats for our experiments. We are ultimately aiming to understand human diseases such as diabetes and dementia. Previous research has shown that there are similarities between humans and rodents in their responses to changes in blood sugar such as the release of hormones (chemical messages) and nerve signals which tend to restore blood sugar back to normal. For mice in particular, there are a number of existing mouse lines with genetic changes which allow scientists like us to examine how different types of cell in the body (including cells in the brain) help control normal body functioning such as control of blood sugar.

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

Animals may be fed modified diets. Some may be genetically prone to become obese with certain diets (like humans).

Some animals may be given injections which destroy insulin-producing cells in the pancreas, leading to diabetes. Like humans with diabetes, they may need to be treated with insulin to control blood glucose. We will use this sparingly so most animals on this licence will not be made diabetic.

Some may be deliberately exposed to doses of insulin to lower blood glucose- typically down into a range similar to that experienced regularly by many people living with (type 1) diabetes. Some animals may experience a combination of these procedures.



Some may undergo brain imaging studies under anaesthetic. In the most onerous protocols, animals may undergo surgery under anaesthetic with brain injections (for example of viruses carrying specific genetic information) and/or implantation (for example tubes which can then be used for delivery of drugs).

Some may have surgery to implant tubes (catheters) into major blood vessels. These tubes are tunnelled around under the skin and covered with a protective magnetic cap at the back of the neck. Generally, rats and mice are social animals and prefer to be in cages with other animals. We will try to keep animals "group housed" with other animals during experiments and the magnetic caps protecting catheters from being damaged by littermates help us to achieve this. Occasionally animals may need to be single housed. After a week to recover, the magnetic cap can be removed and then the tubes used to deliver drugs and take blood samples during experiments. Typically, this will be done with animals free-moving and without handling to reduce stress. To stop the tubes from tangling, they are often physically connected (using a magnet connection that can be easily connected/ disconnected) to a swivel that rotates as the animal moves around the cage.

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 "sub- threshold 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 or for implanting tubes into blood vessels. 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 may undergo repeated insulin injections to lower their blood glucose so that they become temporarily drowsy

For mice undergoing the most invasive studies with surgical implantation of tubes into the carotid artery, we typically see 48 hours of weight loss- usually less than 15% but some may approach 20% weight loss. Some of the mice undergoing carotid artery surgery may reach severe outcomes with continued weight loss and a failure to recover within 2 or 3 days of surgery requiring us to kill them earlier than planned. Rarely, mice may die unexpectedly after undergoing surgery. In our previous licence, around 3.5% of mice undergoing the most invasive studies with surgery to implant tubes into the carotid artery reached a severe outcome requiring us to kill animals or were found dead in the cage (less than 1% of the total number undergoing the most invasive 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)?

Mice: expect non-recovery 5%; sub-threshold 80-82%; mild 2%; moderate 8%; severe 3-



5%

Rats: moderate 100%

What will happen to animals at the end of this project?

- Killed
- Used in other projects

A retrospective assessment of these predicted harms will be due by 30 November 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

Replacement

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

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

We aim to understand how millions of different cells in the body work together in an integrated manner to maintain blood glucose levels and what the consequences are for disease. Currently, there are no computerised ("in silico") or artificial intelligence models in existence that are sophisticated enough to be able to allow us to simulate studies in computer models.

There are some areas though where it is possible to avoid animal work in cells or humans as described below.

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

Cells: Cell work using brain cells (originally from rodent brain but then kept alive for many generations in "cultures") can part replace animal work to give insights into mechanisms by which glucose-sensing cells work for example. We collaborate with a group with expertise in this area. Similarly, it is possible to use stem cells and develop them into other cells including cells similar to the specialised energy- sensing cells we are interested in. We have one of the World's leading groups in this approach working close by us but this is not yet advanced enough to allow examination of glucose-sensing. A more sophisticated approach is to use "organoids" - a type of 3-dimensional culture system which tries to reproduce the complexity of groups of cells arranged into an artificial organ in a test tube.

Organoids have largely been applied in non-brain tissues which are less complex than the brain in structure but researchers are starting to use brain organoids and we will watch developments closely.

Humans: There are some areas where we can and indeed have already studied brain and blood glucose directly in humans.



Experimental Medicine: As an example, in collaborative work, we have found that low blood glucose leads to changes in white blood cells in circulation. These studies were performed in humans with and without diabetes because we didn't need to study the brain. We have also collaborated in non-invasive brain imaging studies in humans such as functional magnetic resonance imaging using high-powered magnets.

Bioinformatics (an approach using very large collections of data): We have been part of a large international study which has pooled together data from hundreds of clinical trials in diabetes. Despite a huge amount of data, this has provided little mechanistic insight into problematic hypoglycaemia- in part because people with major problems with hypoglycaemia are often not included in clinical trials.

Brain tissue: We are looking at available collections of human brains including some with dementia and diabetes. Colleagues have started to examine samples of human brain tissue from brain areas involved in energy balance.

Why were they not suitable?

Cells: As above, cellular work can help reduce the requirement for animal work. Ultimately though, we need to test out the insights gained from individual cells in whole body systems to see how different cell types interact as control of energy balance and glucose metabolism occurs at the “whole organism” level with a number of disparate cell types working together and not simply at a cellular level with a single cell type.

Humans: Our main aim is to investigate and understand human disease and we only use animals to study aspects that cannot be currently examined in humans. This is because we are often studying small populations of specialised cells found deep within the brain which are impossible to access in humans. For example, even modern brain imaging with powerful magnetic scanning in humans can only give information about brain areas containing millions of cells and it is impossible to tell how individual cells are reacting. For bioinformatics and brain tissue banks, the link to clinical data is currently relatively crude (information about types of diabetes, exposure to glucose levels etc) and not yet at a level that would allow us to answer questions about mechanisms.

A retrospective assessment of replacement will be due by 30 November 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your experience?

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 able to gauge a reasonable estimate of the numbers of animals from our



experience with working on broadly similar protocols in previous licences.

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 during tenure of this licence.

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

We 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/3rs-resources/breeding-and-colony-management>).

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). Where possible, we will use randomisation and blinding so that researchers are not aware of whether animals are undergoing control or intervention procedures.

We have shared tissues collected from experimental animals with other groups- for example through the HypoRESOLVE consortium and will continue to do so. We also share surplus animals where appropriate through the University's initiative to limit animal use.

A retrospective assessment of reduction will be due by 30 November 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

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 modified and wild-type mice to allow us to study how brain areas control blood glucose in humans with diabetes. Some mice may undergo injections under anaesthesia into brain areas to allow us to study these specific areas in greater depth.

We will create diabetes in some mice and/or feed them a diet to make them obese to understand how the brain controls diabetes in humans and in turn how diabetes and



obesity can affect the brain. We may also use rats or mice with an inherited tendency to become obese for the same reason.

Some mice and rats may undergo brain imaging studies under anaesthetic which will allow us to examine how brain blood flow changes in diabetes/obesity.

We will mostly use mice in these studies but may use rats where there is a specific scientific justification. For example, the best-described model of dementia developing in rodents with diabetes/ obesity is a type of rat model called the Zucker fatty rat.

A major aim for us is to understand how blood glucose levels are controlled. To examine this in more depth, we will use a variety of methods to assess blood glucose control. We will always aim to use the method with the least suffering for animals that allows us to gain scientific understanding. The methods we will use range from relatively simple injections into the abdomen of glucose (or insulin to lower blood glucose) to see how much and how quickly blood glucose changes through to more elaborate measures where mice will undergo surgery under anaesthesia to place tubes into blood vessels. After a few days' recovery, we can use these tubes to take blood samples and infuse insulin and sugar into the bloodstream of mice without handling or restraining them. This is a technique called an insulin clamp which has been adapted from human clinical research and miniaturised for rodents. Insulin clamps allow us to create very carefully controlled changes in blood glucose and insulin.

The advantage of insulin clamps is that they allow us to perform studies in free-moving animals without restraining them to minimise stress. The major drawback is that the surgery to implant the tubes into blood vessels, especially into the carotid artery (one of the main blood vessels carrying blood to the brain) is difficult. We have described in the section below how we have refined the surgery and anaesthesia. The artery is less than 0.5 mm in diameter and can be damaged during surgery. If we know that it has been damaged, we will kill mice under anaesthetic without waking them up.

Despite these refinements, a small number of animals suffer and fail to recover normal health after surgery. This may include a smaller number who die suddenly and unexpectedly in cages despite appearing to be recovering well. We monitor mice very carefully in the first few minutes of recovery and then daily for the first few days after surgery and kill animals that are not recovering. To date though, we cannot predict the subgroup that die unexpectedly. This is why the protocol where we will implant carotid arteries is a severe one.

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

For "lower species", it hasn't been possible to create easily translatable glucose challenges and measure responses in fruit flies, fish and or nematodes. We are aware that glucose tolerance testing and perhaps glucoprivic challenges are possible in fish although not aware that the latter has ever been utilised and outcomes would be too crude for our purposes (hormone and or behavioural/ cognitive measures). In general, we are largely interested in examining how the brain integrates with peripheral organs in homeostasis so performing studies under general anaesthetic which alters brain physiology would be challenging to interpret and likely need duplicating in awake animals.

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



We have refined surgery with (i) the use of a low-flow vaporiser for anaesthesia which reduces airflow into the lungs along with oxygen monitoring during surgery, allowing more rapid recovery afterwards (ii) using commercial arterial round-tipped catheters and buttons with protective aluminium caps to allow group housing of mice after vascular surgery. The caps are robust enough to allow environmental enrichment including tunnels and are easily replaced if dislodged or removed by researchers for experimental access with a simple magnetic attachment. The round-tipped catheters are more gentle to the lining of the carotid artery and since changing to these catheters, mice are less likely to reach severe outcomes after surgery (iii) animals are carefully monitored in the first 30-60 minutes after surgery and killed if not recovering as described in protocol steps later (iv) we have switched from injectable to oral analgesia after surgery.

We have devised and used a "hypoglycaemia scoring scale" to make certain that blood glucose doesn't go too low in animals undergoing deliberate low glucose exposure.

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

We will apply the Laboratory Animal Science Association (LASA) aseptic technique guidance for surgery (2017-"Guiding Principles for Preparing for and Undertaking Aseptic Surgery") and ARRIVE and PREPARE guidelines.

Where we use food restriction, we will also apply NC3Rs guidance on food restriction to minimise or even avoid restriction completely where possible.

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

Licence holder and lab manager subscribe to NC3R newsletter and receive regular information about advances and/or relevant 3Rs and methodology webinars and Laboratory Animal Science Association (LASA) practical guidance. We will also look for advice from Named Person(s) about advances in 3Rs.

A retrospective assessment of refinement will be due by 30 November 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?



16. Cardiac Conduction System in Health 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

Conduction system disease, Cardiac arrhythmias, Gene therapy, Ageing, Circadian rhythm

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

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

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?

Dysfunction of the cardiac conduction system is an important cause of disability and death in clinical medicine. The aim of this project is to understand the molecular and cellular basis by which the cardiac conduction system becomes dysfunctional (in athletes, in the elderly and in heart failure) and address why this dysfunction is particularly evident at night. This information will allow us to identify new therapies to correct conduction system disease.



A retrospective assessment of these aims will be due by 11 December 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

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 heart has an electrical wiring system called the cardiac conduction system that is responsible for the initiation and coordination of the heartbeat. It is in essence the conductor of the heart's orchestra. The system frequently goes wrong resulting in a slower rate or disordered rhythm which can be life threatening. Importantly, conduction system dysfunction often increases the risk that an individual will develop other serious heart conditions including atrial fibrillation (an irregular and fast heart rhythm) and heart failure. Despite this clinical importance, little is known about the mechanisms that cause conduction system disease and at present the only treatment is the surgical implantation of an electronic pacemaker. This can present problems for the patient and the healthcare provider, and there is a clinical need for improved treatments and risk prediction strategies. Worldwide, over 1.2 million electronic pacemakers are implanted annually and a large proportion of these are to mitigate the impact of conduction system disease in aged and failing hearts. Intriguingly, veteran athletes who have been exercising at a high level for decades are also more likely to need an artificial pacemaker due to conduction system dysfunction. Finally, for reasons that are poorly understood, the dysfunction also follows a roughly 24-hour cycle or 'circadian rhythm' and is most apparent at night.

Our work in animal models over the last 10 years has shown that in endurance training, heart failure and in ageing, the system goes wrong primarily because of a loss of proteins known as ion channels

which are responsible for the heart's electrical impulse. Over the last 5-year period we have made important advances in understanding the molecular events that cause ion channel loss. We have identified that in aged, trained and failing hearts, specific molecules known as microRNAs and transcription factors become activated, and directly reduce ion channel expression. We have also shown that targeting these molecules can repair and restore electrical activity in the diseased cardiac conduction system. Strikingly we have also demonstrated that ion channel levels in heart cells naturally vary over the course of the day due to the rhythmic rise and fall of molecules known as 'clock transcription factors', and the stress hormone cortisol. We have observed that targeting these newly identified molecular pathways prevents not only slowing of cardiac conduction at night but also the



occurrence of other potentially lethal heart rhythm disturbances that occur in the early morning hours.

In the proposed project we will build on these advances and perform studies in mice, in human tissues, in cell lines and in computer models of the heart to improve our understanding of the precise cellular signalling events and the master regulators of ion channel expression levels in the conduction system. We will focus on the role and interaction between microRNAs, transcription factors and other molecules that we have identified to be important in maintaining conduction system function. Using mice as models, we will then test therapies designed to prevent or reverse ion channel loss and conduction system disease in the elderly, in heart failure and in endurance athletes. This work will lead to the development of new treatments directed at the root causes of cardiac conduction system dysfunction and failure. We will also conduct studies to increase understanding of the day-night rhythm in cardiac ion channels, leading up to the preclinical development of a therapy to prevent the occurrence of cardiac arrhythmias at certain times of the day.

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

The major output of the proposed research will be new fundamental insight into the mechanisms that underpin conduction system disease, and improved understanding of the functional effects of specific pathways and signalling mechanisms. We anticipate that our research will lead to the identification of druggable targets for human conduction system dysfunction that occurs as a response to sustained endurance exercise, heart failure and ageing, and new insight into why heart rhythm disturbances occur at specific times of the day. We will communicate our advances through high-quality peer reviewed articles, and through presentation at international meetings.

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

In the short term the proposed work will lead to new ideas and advances in understanding conduction system disease within the cardiovascular community. Some of our approaches, have the potential to yield new 'druggable' small molecule targets to prevent ion channel loss, and this may be of benefit to industry (medium-term). In the long term we hope that our research will directly input into the development of new therapeutics for conduction system dysfunction and associated arrhythmias.

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

We are internationally recognised for the study of the cardiac conduction system and regularly approached for collaboration and training by other groups at our own institution and elsewhere. We share good practise in terms of in vivo techniques and analyses and will continue to do this, and be receptive to new collaborations and sharing of genetically-modified animals generated on this study. We will maximise access to our outputs by publishing in open access journals and depositing datasets (including those with negative outcomes) in publicly available repositories where appropriate.



Species and numbers of animals expected to be used

- Mice: 1700

Predicted harms

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

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

We will use the mouse because it is the best-characterised small animal model of conduction system dysfunction seen in humans, and because we have extensive experience of working with this species. With the exception of studies on ageing we will typically use young adult mice in our protocols. For studies on ageing we will use mice between 20-24 months of age as ageing-associated conduction system dysfunction becomes overt and appreciable at this time point.

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

During this project we will typically model the effects of endurance exercise, heart failure or ageing. Typically, wild-type mice purchased from a recognised breeder, or mice bred in-house to carry a genetic mutation will be group housed with age-and gender-matched litter mates. Mice will be either be

- (i) exercise-trained using a treadmill setup OR
- (ii) subjected to a surgical procedure under anaesthesia in which a suture is placed around the aorta to restrict blood flow. This causes an increase in heart muscle size and heart failure in approximately 6-8 weeks OR
- (iii) aged upto 24 months.

The duration that the animals will be exposed to the effects of exercise, heart failure or ageing will be the minimum required to obtain data on the chronic changes to the cardiac conduction system. For studies of the circadian rhythm, animals may be subjected to a modified 12 h light - 12 h dark cycle in order to study heart function during the animal's active phase (night time) and this is not associated with any adverse effects to the animal.

In some cases, a device used to record the heart rate may be surgically implanted under anaesthesia so that recordings can be taken from the animal (following one week recovery from surgery) without the animal being aware measurements are being made. As in clinical medicine, all surgery will be conducted using general anaesthetics, aseptic techniques, and animals given post-operative pain relief.

Subsequently, studies will be performed to assess heart function. A series of methods similar to those used to assess human heart function e.g. an electrocardiogram (ECG) and



cardiac ultrasound will be used to analyse cardiac function and structure. These analyses may be carried out at a single time point, or at multiple time points to analyse changes over time. In some studies, animals may be terminally anaesthetised and conduction system function assessed in further detail by introducing a catheter into the heart through the jugular vein. On completion of these studies, animals will be humanely killed while remaining under anaesthesia.

In some experiments the impact of an intervention will be assessed. For example, animals may receive therapeutic drugs orally, by intraperitoneal or intramuscular injection or by drug administration in the food or drinking supply. If drugs cannot be administered by these routes, they may be administered by implanting a small device known as an osmotic minipump under the skin under anaesthesia. Blood samples may be collected to analyse circulating biomarkers and/or hormone concentrations.

At the end of each study animals will be humanely killed and tissues harvested for further electrophysiological and molecular study of ion channels and their regulatory pathways.

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

When studying disease, adverse effects are possible, but every effort is made to avoid them.

With exercise training (severity categorised as moderate), mice may undergo temporary psychological and physiological stress during the training period (2 times per day for upto 60 min per session, 5 days per week for up to 6 weeks). Animals will be monitored closely and gradually habituated to the treadmill setup and the training regimen. In our experience >90% of mice quickly adapt to the training regimen and become fitter and sleeker as the training progresses.

Pain from surgical procedures is expected but in our experience, animals make a full recovery from surgical procedures which are conducted under anaesthesia. Pain relief will be administered following surgery, animals are monitored very closely, and humanely killed if humane end points set out in the licence are encountered.

The development of heart failure is the recognised objective of one of the protocols (categorised as severe) and animals may display signs of heart failure including weight loss, reduced activity and increased respiratory rate at 6-8 weeks following surgery. In our current studies, as in human heart failure, sudden death may occur due to a lethal arrhythmia in up to 5% of animals but in this case death is usually instantaneous and unlikely to cause lasting distress or suffering. Deaths may occur during the complex microsurgical procedure used to constrict the aorta but this is under anaesthesia and has little adverse effect on the welfare of the animal.

Normal ageing (classified as moderate) may lead to frailty and increased pathology including tumour incidence. However in our recent and ongoing studies (approximately 50



aged animals aged 20-24 months) no adverse effects necessitating humane culling were encountered.

No adverse effects are expected as a result of genetic modification or cardiovascular assessments (conducted either in the conscious animal or under recovery and non-recovery anaesthesia).

Administration of substances and collection of blood from the tail vein or superficial vessels may cause transient pain and discomfort (<24 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 severities are as follows:

17.6% - sub threshold

47.6% - mild

34.3% - moderate

0.5% - severe

What will happen to animals at the end of this project?

- Killed

A retrospective assessment of these predicted harms will be due by 11 December 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

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 study aims to model human cardiac conduction dysfunction that involves the integrated response of cells and organs. If we were studying biochemical pathways, it may be possible to use a suitable cell line. However, this proposal concerns complex and interrelated systems which cannot be fully recapitulated in vitro or in silico. Thus in order for the results to be extrapolated to human physiology, there is no viable alternative to the use of animals.



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

We routinely use non-animal alternatives such as cell lines and computer models of the cardiac action potential to test the validity of hypotheses, pre-screen compounds and gather preliminary data before commencing in vivo studies in animals. Conduction system cardiomyocytes are not amenable to cell culture, but we have attempted to use cell lines and human induced pluripotent stem cells differentiated into cardiac pacemaker-like cells. We routinely perform in silico studies to understand the role of electrical remodelling in cardiac pacemaking and conduction and contribute to the development of computer models of cardiac electrical activity in collaboration with other researchers.

Over the course of the previous PPL, we have sought and have established links with clinicians to obtain human tissue from patients with conduction system disease. Human conduction system biopsies can only be obtained from the deceased, and the types of experiments that are technically feasible in these samples are limited. The applicant is currently liaising with cardiac surgeons with a view to optimising the collection process of human hearts that are unsuitable for transplant so that conduction system biopsies can be harvested in a timely fashion and utilised for electrophysiological studies in vitro. We are also beginning to assess the feasibility and utility of performing electrophysiological, pharmacological and molecular studies on the day-night rhythm in ion channels in an established cultured human ventricular slice model with a view to replacing the use of animals in certain types of experiments.

Why were they not suitable?

Currently available in vitro and in silico models are poor surrogates of the complex multicellular system that orchestrates electrical activity of the heart. Although they can complement understanding of specific biological pathways, they are not a suitable replacement for understanding disease mechanisms, progression, or for addressing potential treatments for heart rhythm disturbances as they do not accurately represent the complexity of the disease. Human conduction system biopsies can only be obtained from the deceased (typically from failing hearts), and the types of experiments that are technically feasible, and the research questions that can be reasonably addressed in such samples are limited. As such although we aim to use non animal alternatives wherever possible, we cannot envisage completely replacing the use of animals at this time.

A retrospective assessment of replacement will be due by 11 December 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your experience?

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 always take measures to ensure that the minimum number of animals will be used and have developed and refined techniques over many years which enable us to ensure this while achieving accurate and statistically significant outcomes. The proposed numbers have been estimated based on our experiments over the last 10 years. We have carried out these types of studies many times before and are highly experienced in understanding the variability typically encountered and the number of animals needed.

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. Our protocols are structured so that multiple measures can be collected in the same animal (ECG, echocardiography, biotelemetry data, intracardiac measurements) to maximise use and reduce numbers required to answer the scientific questions. 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 generate transgenic lines only if other options (e.g. gene delivery strategies) are unsuitable. Our breeding strategies are optimised and colony sizes reviewed regularly to match with experimental demand. We collaborate widely and routinely share tissues within our institution and also externally. We continuously assess and update our methodology, utilise previously deposited repository data, cell lines and in silico models for hypothesis testing prior to commencing work in animals.

A retrospective assessment of reduction will be due by 11 December 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

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 proved to be an ideal experimental model for studying the cardiac conduction system in health and disease, providing a close replication of human cardiac physiology and cardiomyocyte function. Relevant to the aims of this study, it is amenable to genetic manipulation, its genome has been sequenced to completion and a wealth of genetic resources and tools are available for use in mice, to enable detailed examination of mechanisms underlying cardiac conduction system disease. Over the years, our group has pioneered techniques for the study of the anatomy and physiology of the cardiac conduction system, and we have extensive expertise, technical know-how and equipment to study the mouse, while keeping welfare costs at a minimum. The genetically modified strain we are currently using (and strains that we are likely to use) delete genes only in the heart or in certain types of heart cells which in general allows us to restrict the overall impact to the health of the animal. Our protocols to investigate specific physiological (exercise, ageing, circadian rhythm) and disease (heart failure) contexts have been chosen based on published literature to understand conduction system remodelling while ensuring that animals under investigation experience the least pain, suffering, distress or lasting harm.

Methods: Our general approach is to restrict intervention to the minimum required to achieve our scientific objectives, with a clear focus on animal welfare. We aim to minimise suffering, distress and lasting harm by frequent review of practices, ensuring rigorous training and support of all staff involved in handling animals and conducting experiments. Animal welfare costs are further minimised by applying gold standard approaches that we have further refined over the years. For example, in the study of the effects of exercise training on heart function, we have replaced swim training with treadmill running as this was considered more refined and was associated with reduced stress levels. In our heart failure model, we have identified optimal substrains, ages and weights and we keep within these parameters. This model typically involves surgical ligation of the aorta and falls within the severe category. In this and other surgical interventions (device or osmotic minipump implantation), suffering will be minimised through the use of peri-operative analgesia to alleviate pain, fluid administration, careful husbandry and regulation of temperature post-surgery. We will also minimise suffering by constant and if necessary increasing monitoring frequency following surgery. Where appropriate and in close consultation with the NACWO, we may utilise score sheets to further assess and minimise suffering in these animals. In work on aged mice we have developed refined monitoring



and husbandry practises such as frailty scoring sheets to ensure that they maintain good body condition. In studies of the day-night variation in conduction system function, we may, on occasion, gradually shift the 12h light/ 12h dark cycle. This is not associated with any distress or suffering.

Where appropriate, analyses are carried out while the animal is conscious, for example recording of the ECG using an 'ECGenie recording platform' which leads to minimal stress in the animal and negates the use of anaesthesia or surgical implantation of ECG monitoring devices. Physiological analyses (e.g. echocardiogram, ECG and pacing protocols) are performed under general anaesthetic, in many cases the mouse is under terminal anaesthetic from which it does not recover. Any stress caused by administration of ion channel modulating agents is momentary as the injection is given. We will couple this with careful selection of dose levels to reduce the likelihood of unexpected toxicity, and the application of rigorous and comprehensive humane endpoints. Where appropriate, mini-osmotic pumps will be used to administer agents that modify heart rate and rhythm in an effort to minimise handling stress. Our collaborators have shown that this can lead to highly reproducible and consistent results requiring fewer animals per experimental group.

Our methods will be constantly reviewed and scrutinised to ensure the highest possible standards of animal welfare.

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

Our studies relate to the mechanisms that underpin conduction system function and therapeutic interventions for human conduction system disease, a process which develops over time (weeks- months). This work requires a model that has a cardiovascular system similar to humans and given this requirement, the mouse is the least sentient option. It is not possible to conduct this study in a less sentient species or at immature life stages as this would mean greater separation from adult human cardiovascular biology which would render our findings non translatable. Where appropriate, studies (physiological measurements) will be conducted in terminally anaesthetised animals.

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

The proposed combination of protocols and procedures have been selected because they are extremely well characterised, enable multiple measurements of heart function and maximally refined over many years. We will continue to assess the possibility of further refinement.

We make every effort to ensure the optimal welfare of our animals. Analgesia and anaesthesia are given wherever appropriate, we have written procedures for all our models and protocols and new researchers are trained and signed off as competent by dedicated trainers. In the event that animals show adverse clinical signs after intervention, we will increase the frequency and length of observations and provide supplementary interventions (like extra bedding/mashed food) until the signs resolve. If we decide an



animal is not recovering, appears to be in pain that cannot be controlled or which has significant surgical complications, or whose general health deteriorates, will be humanely killed after seeking advice from the NACWO and NVS.

Over the course of the previous licence we have made a number of refinements to minimise harm to experimental animals

Exercise training

We have acquired apparatus and developed custom protocols to reduce stress during chronic exercise training studies. We have replaced the mouse model of swim training with an intensity-controlled treadmill running protocol. To do this we have purchased state-of-the-art equipment that is now utilised by a number of other researchers.

Refinements to anaesthesia and surgical approaches

- (i) Work as a team during surgery. This has led to a reduction in surgical time and improved recovery. Refined use of pre-op and post-operative analgesia.
- (ii) We have found optimal ages and weights for inducing heart failure and we keep within these parameters.
- (iii) Timing of surgery: all surgery is conducted in the morning and at the beginning of the week to allow frequent monitoring during the normal working week. This ensures that more research staff and animal unit staff are available for advice during the surgical recovery period.

Monitoring

- (i) Detailed study plans are drawn up for each experiment and named persons consulted. This allows us to readily monitor and assess the benefit of each study.
- (ii) Regular appraisal of surgical outcomes.
- (iii) Continually refine the monitoring documentation to aid in assessing mouse welfare.

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

Procedures will be undertaken in accordance with institutional standard operating procedures (SOPs) and guidelines.

The approach to surgical procedures will be further informed by the Laboratory Animal Science Association's (LASA) Guiding Principles for Preparing for and Undertaking Aseptic Surgery (<https://www.lasa.co.uk/wp-content/uploads/2018/05/Aseptic-Surgery.pdf>).

Blood sampling will conform to guidance published on by NC3Rs (<https://nc3rs.org.uk/blood-sampling-mouse>).



During the planning phase of our experiments we will refer to the PREPARE: guidelines for planning animal research and testing (DOI: 10.1177/00236772177248).

When publishing the outcome of our work we will adhere to the ARRIVE guidelines (<https://arriveguidelines.org/>).

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

We will regularly attend biological service unit meetings to keep ourselves updated of new developments and advances in the care and welfare of animals used for scientific experimentation. Our work environment has an established culture of care and we are in regular receipt of the newsletter from understanding animals research and NC3RS. We will continue to stay informed of 3Rs advances through innovations published in the literature, discussions with colleagues at our own and other institutions and through close interactions with the relevant welfare, training and information officers as they oversee in vivo studies.

A retrospective assessment of refinement will be due by 11 December 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?



17. Generation of positive antisera to infectious horse diseases

Project duration

5 years 0 months

Project purpose

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

Key words

Antisera, Horses, Testing, Equine infectious anaemia (EIAV), Equine Viral Diseases

Animal types	Life stages
Horses	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Uses cats, dogs or equidae

Objectives and benefits

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

What's the aim of this project?

To generate positive antisera for validating existing diagnostic testing for one notifiable disease- Equine infectious anaemia virus (EIAV).

To fulfil the requirement of the National Reference Laboratory to generate antisera and immune cells for other equine virus such as Equine Viral Arteritis (EVA) of horses and Western, Eastern and Venezuelan Equine Encephalitis Viruses (WEEV, EEEV, VEEV respectively) as required.

A retrospective assessment of these aims will be due by 19 December 2029

The PPL holder will be required to disclose:



- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

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 organisation is the national reference and testing laboratory for the UK for Equine infectious anaemia virus (EIAV), Equine Viral Arteritis (EVA) and the equine Encephalitides (WEEV, EEEV, VEEV); producing this material will allow the continued trade of horses and other Equidae into and out of the UK and, should it enter the country, allow its eradication.

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

Producing this material will allow the continued trade of horses and other Equidae in the UK and should it enter the country contribute to its eradication. The production of antisera and immune cells is necessary for validating existing and future diagnostic tests to detect the disease in the face of variation of viruses like EIAV.

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

The achievement and maintenance of disease freedom from notifiable equine disease has substantial benefits for the health of all the Equidae in country. Benefits also include the international movement of horses and ponies as well as trade, economic and also personal freedom benefits.

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

The Establishment is the national reference and testing laboratory for the UK for Equine infectious anaemia (EIAV), Equine Viral Arteritis (EVA) and the equine Encephalitides (WEEV, EEEV, VEEV); producing this material will allow the continued trade of horses and other Equidae into and out of the UK and, should it enter the country, allow its eradication.

We have attempted to source this material to avoid the use of animals, but it is not available. Thus, if sufficient material is generated it will be provided to other national authorities who have the same requirement for this testing

Species and numbers of animals expected to be used

- Horses: 9 over a period of up to 5 years

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 not yet possible to produce polyclonal antisera without the use of animals. The antisera



needs to be raised in horses as they are being used as a positive control.

All approaches are to generate antibodies to specific proteins, so field cases are not appropriate. Adult equines are the life stage of choice as this work requires a fully competent immune system.

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

The horses will receive an initial injection of antigen (inactivated or subunit and not infectious) plus adjuvant (e.g. montanide). This will be followed by a small test blood sample and a booster injection at around 3 to weeks. Depending on the titre the animal may be subsequently bled and boosted up to 5 additional boosters.

Since the antisera generated need to be highly specific and shall serve as positive controls in various tests (serological testing is carried out by test such as ELISA, Western Blot and Agar Gel Immunodiffusion Assay (AGID)), the resulting sera need to be highly positive across all applicable tests. When this is achieved a larger volume of serum will only be required a maximum of two times in no less than 3 months.

We will also use this approach to generate anti-viral T cells that may hold the answer to future next generation tests for virus infections. To secure those cells blood samples (unclotted blood) will be taken separately and peripheral blood mononuclear cell (PBMC) safely stored (frozen). We will further isolate the B cells for these cells, which will us allow to clone monoclonal antibodies (Abs) against the viruses and viral proteins in the future without the need for additional animals.

At the end of all procedure, the animals will be assessed by a veterinary surgeon to determine if they can be re-homed with the knowledge of the life history of the horses. If it is deemed that they cannot be re-homed (for example if they remain seropositive for a notifiable disease or if they have to be bled out to maximise the harvest of antibodies), the animals will be euthanised and the maximum volume of blood and tissues will be taken after death.

Initially the antisera and cells will be for Equine infectious anaemia (EIAV) but Equine Viral Arteritis (EVA) of horses.and/or equine Encephalitides (WEEV, EEEV, VEEV) may also be done if there is requirement from the NRL as necessary to undertake its function.

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

The severity limit is mild, and we fully anticipate it to remain mild following the experience gained over the last 5 years on the previous licence . Non-infectious proteins and adjuvants will be used. The antigens will be purified to the best possible way to avoid the contamination by danger signals that lead to local inflammatory reactions. In turn we will use the most advanced adjuvants to ensure we need to apply as few injections with the least side effects possible.

Occasionally a slight swelling (hematoma) around the blood sampling site can be seen (rare).

Animals will be observed closely by trained and competent animal care staff, Named Animal Care and Welfare Officers and/ or Named Veterinary Surgeons after blood sampling and/or injection for any signs of swelling, pain or discomfort.



Moderate swelling, myalgia and discomfort at the injection site have been observed due to the animals' increased sensitisation to the antigen in the past . The use of prophylactic analgesia is recommended to avoid and mitigate 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)?

Mild, 100% of horses.

What will happen to animals at the end of this project?

- Kept alive
- Rehomed
- Killed

A retrospective assessment of these predicted harms will be due by 19 December 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

Replacement

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

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

It is not yet possible to produce polyclonal antisera in vitro as the complexity of the horse immune system is required, particularly as the reagents are used in the diagnosis of disease in animals and high specificity is required.

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

Phage generated non-animal antibody generation were considered but rejected for reasons given in the next section.

The use of non-animal alternatives will be reconsidered as the study progresses via exploring new scientific data, engaging with companies developing in vitro methods, consulting 3Rs centres and in- house expertise.

The establishment is committed to use alternative technologies to replace antisera where possible using non-animal derived antibodies.

Why were they not suitable?



A polyclonal antiserum (with possibly >100 different monoclonal Abs making this up) is currently not replaceable by alternative, non-animal techniques and it is not foreseeable when oligoclonal Ab mixtures will be accepted in diagnostic assay validation. This and the variability in the virus, described above means that non-animal derived antibodies are not considered suitable at this moment. To ease the transition and create further resources to generate antibodies by molecular means we are planning to store the B cells (Plasma cells) specifically from which Abs can subsequently be cloned.

A retrospective assessment of replacement will be due by 19 December 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your experience?

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?

9 horses will be used in total.

3 for each of the 2 EIAV antigens & 3 for a subsequent antisera generation

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

Due to the greater inherent variation in EIAV work, three horses per antigen will be used. This is due to the immunologic reaction to inactivated/killed vaccines and proteins is variable depending not least on the antigen processing and presentation machine of the immune system. The way antigens are recognised is ultimately determined by the Major Histocompatibility Complex (MHC) proteins that each recognise a limited set of peptides from proteins. Each individual is therefore equipped with a set of genes (around 6) from maternal and paternal heritage.

Breeding is limiting the variability of the MHC while genetic variability of the virus is intended to escape the presentation by the MHC. It may safely be assumed that every animal will recognise proteins of EIAV in some form, but not all in the same strength not quality. To capture the host variability we will use three horses of different breeds. The different antisera resulting will allow us to validate the tests taking this variation into account.

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

These reagents are also supplied to several research groups therefore minimising the numbers of animals required. For both antisera and antigen production the key to reducing



numbers is to maximise the amount and titre of the material collected from each animal.

A retrospective assessment of reduction will be due by 19 December 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

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 antisera needs to be raised in horses, as they are being used in diagnostic tests for horse diseases. Raising in other species would lead to reductions in sensitivity and specificity due to genetic differences in their immune systems.

The use of dead antigen and adjuvant avoids using infectious agents which would cause disease and is the most refined method known.

Prophylactic medication will also be administered at the time of inoculations to minimise swellings and discomfort in the injection site.

We have 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.

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

To produce antisera a competent immune system is required, this involves using fully developed animals with associated sentience.

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

As well as pre-start meetings involving the NVS, NACWO and animal care staff to ensure current knowledge is brought to bear, all projects are followed up by a wash up meeting. All aspects are discussed, was the project a success, what went well and if there was anything that could be done better. If there are any suggestions for refining the procedure they will be considered and if appropriate, incorporated into the protocol.

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

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?

Regular updates from the NC3Rs webpage (<https://www.nc3rs.org.uk/>) as well as keeping up to date with other accessible sources of information with advances in the field.

I have regular contact with the NVS, NACWO, NIO and ensure I am kept up to date with current guidelines and practices by having direct conversations before at pre-start meetings, during studies and regular welfare meetings. Our network of veterinary excellence that supports all animal work at the organisation will guide where modifications may be developed that apply to this license.

A retrospective assessment of refinement will be due by 19 December 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?



18. Mechanisms of Cardiovascular Ageing and Calcification

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 dysfunction, Ageing, Calcification, Cardiovascular remodelling and Heart failure, Therapy

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

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

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 further our understanding of the processes that underlie cardiovascular diseases (CV). This project is to identify key mechanisms involved in the development of cardiovascular dysfunction, ageing and calcification as well as heart failure, with potential to identify novel targets for new therapies.

A retrospective assessment of these aims will be due by 21 December 2029

The PPL holder will be required to disclose:



- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

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 relevance of the cardiovascular system for human health is highlighted by the global death statistics: Almost a third of all deaths are caused by CV diseases such as ischaemic heart disease and stroke. Currently there are no ultimate treatments to restore cardiovascular function. Thus, if molecular targets that reduce disease progression and/or improve cardiovascular cell function are identified for therapy development, they would have a huge impact on the welfare of society. Alterations (known scientifically as mutations) in a gene known as *LMNA* which provides instructions for the formation of the proteins lamin A/C can lead to more than 20 disorders – collectively these diseases are known as laminopathies. Examples include dilated cardiomyopathy (DCM) and several early ageing disorders such as Hutchison-Gilford Progeria Syndrome (HGPS). More specifically, HGPS is caused by a build-up of the toxic protein prelamin A. This leads to premature atherosclerosis (the build-up of fat and cholesterol plaques in blood vessel walls) and calcification (the build-up of calcium ions within the vessels). Both are associated with stiffening and may lead to premature deaths for example from myocardial infarction and stroke.

Age is a potent risk factor for both atherosclerosis and calcification – the ultimate outcome of the ageing process. Critically, prelamin A accumulation is observed in human cardiovascular tissue from old patients but also in young ‘prematurely’ aged chronic kidney disease (CKD) patients. This suggests prelamin A accumulation may be a unifying mechanism for calcification in many diseases as well as in conventional ageing.

Vascular calcification is a serious and common clinical problem in atherosclerosis, diabetes, CKD and ageing and is set to become increasingly prevalent. It is an independent risk factor for CV mortality in all disease contexts and calcification is directly causal in the induction of CV events such as ischemia, heart failure and arrhythmia. Currently there are no treatments to prevent or regress vascular calcification. In patients with heavily calcified arteries even standard treatments such as statin therapy do not significantly improve CV outcomes. Therefore, there is a serious unmet clinical need to understand the molecular mechanisms driving the calcification and ageing process and to identify novel treatment strategies.

Additionally, there is need to develop techniques capable of identifying early markers of vascular remodelling events that precede calcification. Our work has identified vascular smooth muscle cell (VSMC)-specific mechanisms of ageing, which accelerates calcification and is caused by nuclear lamina disruption. We have now developed novel animal models of premature ageing and calcification that reiterate the phenotypes seen in man enabling us to define new pathways and test novel therapies.

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



This work is expected to significantly increase biological and disease-related knowledge and to be highly relevant to the treatment of ill health. The project should substantially increase our understanding of the mechanisms involved in Cardiovascular dysfunction, Ageing and Calcification and the progression to heart failure, particularly lamin A/C and precursor prelamin A as well as associated nuclear envelope proteins e.g. nesprin family that influences multiple processes in remodeling vessel and heart. We aim to identify specific drivers of adaptive versus maladaptive cardiovascular remodelling, as well as pathways that promote reverse remodelling, which can form new therapeutic targets. By elucidating underlying mechanisms and by undertaking initial experimental studies *in vivo*, this research may provide the basis for devising novel therapeutic strategies for human cardiovascular disease. This is vital to achieve in the longer term because vascular calcification and heart failure imposes a major disease burden on a significant section of the adult population and leads to very substantial costs for the health service both in the UK and worldwide. Furthermore, there is a compelling need to identify more effective treatments for this condition. Outputs will include publication in open-access peer reviewed journals, poster and oral presentations at conferences, and novel data that forms the basis for development of new treatments.

As our projects have a strong translational aim (e.g. to develop new drugs and treatments for cardiovascular disease), we already have ties, and plan to expand them in the future, with pharmaceutical companies for the subsequent development of the therapeutic leads we identify.

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

Results obtained in this project will be published in open-access journals continuously through the 5- year period and will therefore add to the body of publicly available knowledge for the wider scientific and clinical community. Findings will also be disseminated to the scientific and medical community by presentations at seminars and conferences. They will be of value to other research groups working in the field of cardiovascular dysfunction, ageing and calcification, including groups developing new therapies. A significant focus of the project is to identify new therapeutic targets and potential therapies. We therefore expect that in the longer-term this work will benefit patients with ageing, calcification and heart failure and have wider societal impact by reducing the consequences of this debilitating condition.

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

All publications will be open access. Both positive and negative findings will be published. ARRIVE guidelines will be followed for publications to maximise *in vivo* information in research articles. The PREPARE guidelines (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) will also be followed. New findings will be disseminated at national and international conferences and seminars. Our lab has extensive national and international collaborations which will further enhance dissemination. We also host visits from other researchers for them to obtain direct exposure to our work.

Species and numbers of animals expected to be used

- Mice: 14500
- Rats: 500



Predicted harms

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

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

The study will be performed using mice and rats for several reasons. Information on the role of new signalling pathways involved in vascular ageing, calcification and heart failure that are obtained in rodents is generally translatable to the human disease because of conservation of key pathways in mammals. Methods to induce experimental models of human disease are available and established in these species as are state-of-the-art methods to quantify the *in vivo* phenotypes relevant to vascular ageing, calcification and heart failure. Aortic constriction models are a severe disease model to mimic human pathology of pressure overload, especially suitable for the study of the transition from cardiac remodelling to heart failure.

Genetic alterations that allow the study of specific biochemical pathways in the animal and the role of such pathways in disease are readily feasible. The majority of work will be undertaken using young adult mice but a proportion of animals will be allowed to age up to 2 years in order to observe longer term effects that may occur without surgical or pharmacological induction of stress. Vascular calcification is an age-associated pathology and therefore to model this appropriately in animals we need to study ageing mice. It has been estimated that a 24 month old mouse is equivalent to approx 70 human years so we do need to go to this age in the mice. Critically we have a model of vascular calcification where the calcifying phenotype only begins at 18 months so in order to study this pathology we need to take measures at least to 22 months due to variability between animals. Importantly vascular calcification in isolation does not have any significant adverse health effects in the mice.

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

Typically animals in this project will undergo pathological cardiovascular remodelling induced either by surgery to denude the carotid artery or to promote cardiac remodelling in response to pressure overload by aortic constriction, or drugs or physiological remodelling in response to exercise.

Genetically altered animals (GAAs) will be used to investigate the effects of specific proteins of interest. Cardiovascular function will be serially investigated (non-invasively) and substances may be given to elucidate the mechanisms involved.

Additional *ex vivo* analyses will be performed after killing the animal at the end of a protocol. Typically animals are followed up for a maximum of 3 months although a percentage that have not undergone surgery or pharmacological interventions will be monitored for up to 2 years. The number of procedures will be kept to the minimum necessary to pursue the main objectives.

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

The majority of mice (>95%) will not develop any harmful phenotype or the phenotype will be mild. Only in a minority of cases the genetic modification of a gene (e.g. prelamin A) can lead to a phenotype causing increase severity. We currently have only one strain of



mice that develops symptoms. This is the Prelamin A-SM22 knock-in line (LMNA) that develop gut problems at 7 months due to an off target effect. These mice are routinely culled at 5-6 months before this phenotype can develop. Signs of this gut phenotype include hunched and scruffy appearance. If this is observed at any point the mice will be culled by schedule 1.

Ageing mice may develop dermal, ocular, oncological, nephrological, cardiac or inflammatory conditions and age-related tumours, reduced activity and weight loss. Specifically, animals with a C57BL6 lineage may develop ulcerative dermatitis as they age. Expected impacts are the gradual development of vascular ageing, calcification and heart failure, and the expected effect of the experimental therapies tested is to prevent this outcome. Appropriate analgesia will be applied to mitigate post-surgical pain. Adverse effects associated with cardiac disease include changes in blood pressure, respiration, cardiac output, inflammation, and may cause discomfort, pain or distress. In rare cases, animals may experience severe adverse effects associated with procedures (eg. carotid artery injury, aortic restriction) performed in the study include abnormal heart rhythms. Any animals that do develop such signs will be promptly managed as outlined under the individual protocols or will be culled as appropriate so that the duration of such effects is expected to be less than 1 day. There may be a small proportion of early peri-operative complications that will either be successfully and quickly resolved or require early culling, so that the duration is expected to be less than 1 day. A small percentage of animals that undergo surgery for aortic constriction may develop a sudden onset of heart failure. The predicted mortality in this instance may be up to 10% for carotid artery injury and up to 25% for aortic constriction.

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

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

Animals in protocols with no surgery will experience a mild to moderate severity (e. g. ageing mice). The majority of animals in protocols with surgery will have a moderate severity, as factors such as pain relief and good aseptic technique will mitigate against a severe severity. These animals will be closely monitored and additional provisions such as supplemental heat, access to food and water and painkillers will be provided to minimise adverse effects. Cardiovascular procedures such as induction of vascular denudation and carotid artery ligation have a less than 10% probability of mortality due to surgical complications. Cardiovascular procedures to restrict blood flow through the aorta, are associated with an overall mortality of up to 25%. Of this the predicted mortality due to heart failure in response to the surgery is up to 10%. Most animals that will exhibit peri-operative adverse effects are expected to be identified during the surgical operation whilst under anaesthesia, and will therefore not be recovered, but instead culled under anaesthesia. However, a small percentage of animals that undergo surgery for the acute form of aortic constriction may develop a sudden onset of heart failure.

Animals that develop such early complications will be culled. However, the occurrence of severe clinical signs of heart failure is not an intended endpoint of any of the studies.

What will happen to animals at the end of this project?

- Killed



- Used in other projects

A retrospective assessment of these predicted harms will be due by 21 December 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

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 vascular calcification and cardiovascular ageing as well as cardiomyopathy and heart failure are complex disorders involving many organs in the body, there is no feasible alternative to the use of animal models. Vascular calcification and ageing and also heart failure are complex chronically developing conditions, involving interactions among multiple cell types and organs and characterised by changes in blood pressure, cardiac output, tissue perfusion, metabolism, inflammation, matrix remodelling, energetics and cell death. Although the development of organs-on-chip (OoC) has revolutionised in vitro cell-culture experiments by allowing a better mimicry of human physiology and pathophysiology, especially to study a specific mechanism, but currently there is still a challenge for cardiovascular OoC platforms to reproduce the chronic aspect underlying the progression of cardiovascular diseases as a whole. Therefore, there is no suitable alternative to animal models for studying these complex and chronically developing conditions.

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

The focus of our laboratory is always to use human models where possible including tissue samples, cultured primary human cells and ex vivo organ cultures of blood vessels. In addition, we have developed 3D spheroid cultures of human cells to study calcification. Importantly we use these models to test drugs and for high throughput screening of potential therapeutics. However, none of these methodologies enables us to understand the physiological impact of the disease processes we study in terms of the cardiovascular system as a whole and beyond. Therefore, animal models are the only avenue, at this stage, that can give the physiological information and tissue interaction data that is required. Use of a mammalian model is essential as other model organisms such as zebrafish do not develop chronic age-associated pathologies and cannot be used to measure key physiological read outs such as blood pressure and so cannot be used as an alternative. Similarly, computational modelling cannot predict complex tissue interactions or physiological changes although we do use human databases to verify any targets we identify in human populations before considering using an animal model.

Why were they not suitable?

These models are unable to fully recapitulate the integration and interrelationship between different cell types in the vasculature, between the vasculature and the heart, and between



the cardiovascular and other body systems - all of which are important in atherosclerosis, vascular ageing and cardiomyopathy and heart failure.

This integrated picture which directly impacts on the clinical presentation of the condition cannot be studied in cell culture studies nor is it amenable to computational modelling due to the numerous uncertainties/unknowns regarding interlinked mechanisms.

Despite the inability of cell culture studies (including human primary aortic cells and induced pluripotent stem cell-derived cardiomyocytes, and engineered human tissue) to model chronic changes and interactions among multiple body systems in vivo, these in vitro studies will be utilised to determine which mechanisms need to be pursued via in vivo studies, thus to provide a guided approach and to limit animal usage.

A retrospective assessment of replacement will be due by 21 December 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your experience?

Reduction

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

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

The experimental design and analysis methods are based on careful consideration of statistics, power analyses and good laboratory practice and have undergone stringent review as part of the grant-awarding process. Individual experiments generally involve factorial design to maximise the information obtained from the minimum resource. The majority of measures are quantitative and suitable for statistical analysis. Comparison between groups will be made by 1-way analysis of variance (ANOVA) or 2-way repeated measures ANOVA followed by appropriate post hoc testing. The exact numbers of animals required will vary with specific experiments and the estimates of coefficient of variation for specific outcome measures but will follow this general principle. Total numbers are also based on breeding considerations for gene-modified animals, eg. for protocol 2, the numbers cover phenotyping the new lines and also ageing and diet mice. There are a large number of characterizations required. For qualitative experiments (e.g. immunohistochemistry), the amount of material required will be the minimum necessary to provide an adequate description.

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

We have implemented imaging methods (e.g. high-resolution echocardiography and cuff blood pressure measurement) that allow non-invasive serial assessment during the development of cardiovascular dysfunction and heart failure in the same animal, which significantly reduces the numbers required.



Serial assessments also reduce experimental variability by allowing comparisons at different time points in the same animal. At the end of the experimental period, when animals are sacrificed, we have developed efficient protocols that ensure the maximum possible ex vivo readouts can be obtained in each animal (e.g. protocols for aorta and heart dissection to allow histological analysis, RT-PCR and immunoblotting all from one aorta and heart and muscle). This also significantly reduces the total numbers of animals required.

We will continue to take advantage of the experimental design tools to keep numbers low e.g. NC3Rs. Online advice resource portal. <https://www.nc3rs.org.uk/topic-specific-resources-0> . The experimental design assistant will also be used.

Sample sizes for most quantitative experiments will be set by power analysis using a significance level of 5% and a power $\geq 80\%$. For example, in a four-group experiment where a difference between groups of at least 25% needs to be detected, if the coefficient of variation is 15% then about 8 animals/group would be required.

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

Breeding of experimental animals will be set up to ensure that litters contain both test and control animals to ensure that the least number of animals are bred for any specific experimental purpose.

Principles of good experimental design will be followed to ensure clear answers to questions being addressed while using the minimum number of animals using power calculations and any previous knowledge from our own studies or the literature. For many studies, non-invasive techniques that allow serial assessment of cardiovascular and muscle function will be used, allowing reduction in numbers. This is especially valuable when assessing the impact of medicines aimed at preventing or slowing calcification and heart failure. Where possible, additional information will be obtained from studies in cultured cells. For GAAs, where suitable lines already exist (established by searching databases), animals will be obtained from the relevant supplier.

If novel therapeutics first identified in high throughput screens using human cells and retested in human organ cultures are to be tested in animals the literature will be reviewed and the drug company source approached to ensure that the most up to date knowledge on drug dosage and administration is followed. A small pilot study will be used to determine the impact of any new drug on animal welfare before continuing to a larger powered study.

A retrospective assessment of reduction will be due by 21 December 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

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.

Small animal models (mice and rats) will be used.

The study will be performed using mice and rats because the experimental models mimic human disease, and all relevant methods and techniques are successfully established in these species. Genetic alterations are readily available in these animals and allow the study of specific biochemical pathways in the animal with a view to understanding their role in the disease and interfering with them to provide new treatments.

Mice will mainly be used. The major advantage of using mice is the wide availability of GA lines or the ease of their generation, allowing the impact of specific genes to be examined far more specifically than achievable with most pharmacological tools. Despite their small size, state-of-the-art techniques (e.g. high-frequency echocardiography, MRI, telemetry) have been implemented to assess rodent cardiovascular structure and function in an analogous manner to humans. Models of vascular remodelling and heart failure in rodents are well established and characterised. There are some recognised differences between rodents and humans (e.g. in the heart rate and rate of progression of cardiovascular disease).

Nevertheless, rodents have proven to be a very useful species in which to undertake cardiovascular studies (complemented by work in vitro and in human settings where feasible) and there are striking examples of new therapies that have resulted from initial studies in rodents e.g. Farnesyl transferase inhibitors, a class of drugs used in patients to reduce the risk of mortality in Hutchinson-Gilford progeria syndrome. Rats will be used less frequently; their larger size may make them a more suitable model for some studies involving gene transfer or in cases where experimental techniques/reagents are better established in this species.

Choice of models and methods

The models to be used to induce cardiovascular ageing and remodelling mimic the major causes of human vascular calcification and heart failure and are all well established and validated in the published literature.

Carotid artery injury is to induce a de-endothelialisation, mimicking vascular lesions after balloon denudation or stent implantation in humans.

Aortic constriction is the most widely used model to induce pressure overload. A refinement we have introduced for thoracic constriction is the use of minimally invasive surgery without open-chest surgery, which reduces mortality.

The methods to be used to obtain in vivo experimental measures are the most refined available for the assessment of cardiovascular structure and function in rodents. We will use state-of-the-art echocardiography and imaging methods. Haemodynamic assessment performed as a terminal procedure again uses "gold-standard" pressure-volume analysis



methodology.

A class of treatments that will be administered without surgical procedures will be pharmacological inhibitors and viral vectors. These are viruses that have been disabled of their harmful properties and are used as vehicles for the delivery of genes. The vectors we will use in most of the cases are based on a virus named adeno-associated virus (AAV). This is a small virus, which does not cause any disease and can enter cardiovascular muscle cells at high efficiency. Some of our studies will also involve the development of treatments that can improve the efficiency of these vectors themselves.

All surgical procedures under all Protocols will be conducted under aseptic conditions to at least the published Home Office minimum, following guidance published by LASA as applicable by staff who hold appropriate PIL categories and have been signed off as being fully competent, with appropriate analgesia, the highest levels of post-operative care standards as set out in the Home Office, including additional monitoring provided following surgical procedures and experimental use, and also appropriate veterinary consultation. In the first 24 hours after surgery, animals will be closely monitored at frequent intervals during this period. Careful attention will be paid to heating, analgesia, body weight, surgical wound-sites, hydration, and signs of pain or distress (signs of pain and distress include lethargy, piloerection, hunched posture, vocalisation) - animals will be given analgesia in line with a regime agreed in advance with NVS. Animals will be reviewed at the end of the working day on the day of surgery and any that have not fully recovered will either be killed by a Schedule 1 method or a programme of more intense monitoring will continue for following 24 hours until the animals recover.

Continued post-operative support when required will consist of the use of heat, analgesia, hydration (water jelly), wet mash as necessary. After initial recovery from surgery, adverse effects may include wound infection, which is rare. Any animal showing swelling, redness or discharge at the operation site but is otherwise well may be treated by minimally invasive methods on advice of the Named Veterinary Surgeon (NVS). The animal will be killed if no improvement is seen in the first 24 hours of treatment or if its condition deteriorates before then. In the case of wound dehiscence, uninfected and minimally inflamed wounds may be reclosed on one occasion within 48 hours of initial surgery. Where absorbable skin sutures are used, sutures will not be removed unless absorption is incomplete and seems problematic for the animals, after 14 days; in this very rare instance light inhalational anaesthesia may be used for restraint where usual methods of restraint are likely to cause undue stress. We will work closely with the NVS and will seek advice on animals whose welfare is giving cause for concern.

During the chronic progression to vascular ageing and calcification in all Protocols, animals will continue to be carefully monitored and any that are in a poor clinical condition will be culled within 24 hours if there is no improvement after intervention.

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

The development of vascular ageing, calcification and heart failure with cardiovascular remodelling is a chronic process that is not possible to achieve in the short time span of a terminally anaesthetised animal. We need to use mammals which closely represent the human condition and that are representative of the complex interactions that occur between body systems. Small rodents will be used, mainly mice. The major advantage of using mice is the wide availability of GA lines or the ease of their generation, allowing the impact of specific genes to be examined far more specifically than achievable with most



pharmacological tools.

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

All surgical procedures under all Protocols will be conducted under aseptic conditions, with appropriate analgesia, the highest levels of post-operative care and appropriate veterinary consultation. All surgical techniques are kept under constant review in order to use the most refined methods, inhalational anaesthesia is the default method which is a refinement that allows for improved recovery times from anaesthesia. Surgery for thoracic aorta constriction utilizes a minimally invasive method without the need to open the chest cavity this is a major refinement which reduces mortality compared to the open chest method. A recent refinement in all surgical techniques is the use of surgical glue to minimize wound dehiscence. Post recovery refinements include the use of gradient heated recovery areas that allow the animal to move away from the heat source. As the major component of mortality or expected side-effects are in the first 24 hours after surgery, animals will be closely monitored at frequent intervals during this period. Animals will be reviewed at the end of the working day on the day of surgery and any considered likely to die overnight will be euthanised. Any animal that is observed to be in pain or distress will receive analgesia via a predetermined regime agreed in advance with the NVS along with other supportive measures including warmth and wet mash. In the days after surgery animals will be monitored at least twice daily. Careful attention will be paid to heating, analgesia, body weight, surgical wound-sites, hydration, and signs of pain or distress.

Since cardiovascular ageing, calcification and cardiomyopathy and heart failure develop slowly, disease progression needs to be followed for several weeks. The development of heart failure may be associated with loss of weight, listlessness and rapid breathing in the late stages, which will be closely and regularly checked and monitored during the study. However, animals will not be allowed to progress to late stage heart failure. Any animal showing signs of heart failure such as listlessness and rapid breathing will be humanely killed. Any clinical problems will be dealt with in consultation with the veterinary surgeon. Animals will be humanely killed at a pre-determined endpoint or at the end of the study, whichever happens first. In addition, aged animals will be weighed regularly starting from the age of 6-12 months, depending on the strain and examined bi-weekly for the formation of tumours and examined for signs of ulcerative dermatitis. Aged animals with a weight loss of 20% will be culled. Aged animals with tumours that exceed 1.1cm and/or affect mobility will be culled. If ulcerative dermatitis causes more than mild discomfort the animal will be culled. Animals that undergo exercise regimens will be carefully monitored to avoid exhaustion, data will be regularly reviewed so that duration and frequency of exercise regimens can be reduced where possible.

In all cases, data output will be kept under regular review and regimens reduced/refined wherever feasible, where it does not affect robustness of the results, this includes the use of Sham surgeries.

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

The NC3R's, publishes several resources including the ARRIVE guidelines (Animal Research: Reporting In Vivo Experiments), and the EDA (Experimental Design Assistant) which are made available to researchers throughout experimental design processes and reviews, and Tech3R's for those carrying out regulated procedures and animal handling.



The PREPARE guidelines (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) will also be followed. The LASA Good Practice Guidelines on Administration of Substances (techniques for dosing) will be followed. The Home Office Animals (Scientific Procedures) Act 1986 (ASPA) and Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes (published 2014) are used to ensure legal compliance with the Standard Conditions of all license holders and animal users.

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

All researchers and animal users will be kept up to date on advances by journal reviews, reading published information or updates, and by systematic review of processes with implementation of any appropriate refinements or improvements where possible and required. Training and additional resources will be made available to all staff throughout the project.

We will monitor the NC3R's website, receive guidance from AWERB and keep up to date with published literature. Good communication will ensure cascade of information to PIL holders, this will include discussion of the 3Rs with colleagues.

A retrospective assessment of refinement will be due by 21 December 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?



19. Untangling mechanisms of neuron-glia-vascular communication in the nervous system

Project duration

5 years 0 months

Project purpose

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

Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Neuron-glia-vascular interactions, Epilepsy, Dementia, Multiple Sclerosis, Hypoperfusion

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

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to better understand how the brain works and how this goes wrong in brain diseases involving damage to special cells in the brain called glial cells.

These glial cells are important for normal brain function and die in diseases such as Multiple Sclerosis, Amyotrophic Lateral Sclerosis, Epilepsy, Periventricular Leukomalacia, Periventricular Asphyxia, Dementia, and Stroke. We want to know how the cells die and



whether we can prevent damage to these cells by adding drugs or medicines.

A retrospective assessment of these aims will be due by 18 December 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

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

Why is it important to undertake this work?

By the age of 60, almost all people have evidence of brain damage caused by a decrease in blood flow to the brain. In severe cases, this brain damage causes loss of brain function called dementia. Currently, there are 45 million people living with dementia but this number will increase substantially in the next decade. Therefore, we must understand how brain damage occurs and how to stop it.

Unfortunately, we still do not fully understand how the brain works, and therefore we do not know what is changing or lost during diseases. The work described here is important because we aim to understand how each cell type functions together in the brain, how blood flow constrains brain function and is linked to epilepsy, and how a decrease in brain blood flow leads to brain damage.

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

We will discover mechanisms that control brain activity which are changed in neurodegenerative diseases such as Multiple Sclerosis (loss of glial cells in the brain caused by immune cell attack), Amyotrophic Lateral Sclerosis (genetic alterations in glial cells that lead to loss of brain function), Stroke (loss of blood flow that leads to death of brain cells), Hypoperfusion (decrease blood flow, e.g. caused by a heart attack), Periventricular Leukomalacia (brain damage caused after difficult births), Perinatal Asphyxia (loss of oxygen during a difficult birth), Cerebral Palsy (resulting brain damage caused by a difficult birth) and Dementia (loss of brain function occurring normally during aging). All of these diseases have links to changes in energy supply to the brain. By knowing the mechanisms involved, we will then provide information about targets and test therapies with the aim to improve symptoms, slow progress or halt disease processes.

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

Currently, there are the following numbers of people with diseases which may be helped by this research:

Worldwide, **perinatal asphyxia** is encountered amongst 6–10 newborns per 1000 live full-term births (2022; doi: 10.1371/journal.pone.0262619).

A total of 2.8 million people are estimated to live with **Multiple Sclerosis** worldwide (35.9 per 100,000 population) (2020; doi: 10.1177/1352458520970841) and 1:500 in the UK (MSSociety.org.uk).



In the UK there are approximately 1.3 million people living with **stroke** (2023; NICE). As babies are increasingly surviving early and difficult births there is a rising incidence of **periventricular leukomalacia** (white matter damage) observed with **cerebral palsy** (reviewed by <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8042490>).

White matter damage is seen in all brains after 60 years, and this is correlated with cognitive deficits and **dementia**. Currently, there are 15 million people in the UK over 60, and 1.6 billion in the world.

This number is set to increase over the next decade and we will all benefit from preventing this damage.

Benefits to human health are the most obvious however all of society will benefit from treatments due to the care and financial burden on us all.

This knowledge will also benefit fellow scientists who will build upon our findings to decipher mechanisms and provide targets for therapies.

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

We will publish in high-impact papers and present our work at every opportunity. As we do currently, we will also collaborate with many partners and rigorously publish our methods and negative data.

Species and numbers of animals expected to be used

- Mice: 9000
- 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 have chosen rodents (mice and rats) because they are mammals with a close physiology to humans. They obviously lack our complete cognitive capacity, however, individual cell types work in a very similar way. We can use rodents to mimic diseases found in humans. As rodents have been used for these disease models for some time, we can build upon and compare our results with those published. Where possible we validate our findings using cell cultures from human cells. However, we cannot just use cell cultures as cell lines that are supposed to mimic (say) neurons or oligodendrocytes will never recapitulate the intricate arrangement of the extracellular space that occurs in vivo. We look at young neonatal mice to understand physiology during development (e.g. pathological mechanisms activated during perinatal asphyxia), and adult mice (up to 1 year) to study pathology found in adults.

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

In order to study pathology caused by hypoperfusion in neurodegenerative diseases, the mice will undergo one or more of the following:



Mouse colony breeding and maintenance: we will breed genetically altered mice. These mice may be culled via Schedule 1 so that tissue can be analysed, or they may move on to another protocol. Some genetic alterations may increase epileptic activity in these mice.

Administration or injection of substances: we may inject substances to achieve one or several of the followings: (1) Invoke genetic alteration of therapeutic targets; (2) Fluorescent indicators or tags; (3) Evoke epilepsy by injecting a subthreshold dose of an epileptogenic agent; (4) Modify propensity to seizure (these will not directly cause epilepsy, but they will increase the likelihood of seizure if they are administered before an epileptogenic substance); (5) Modify cell function; (6) Systematic change (e.g. Changing of light/dark cycles, demyelination, reduction in blood flow or change in inspired oxygen); (7) Vehicle substances to compare with control animals.

Tissue extraction: Some of the mice may be culled via schedule 1 so that tissue can be analysed.

Surgical procedure: In some protocols, we may perform a surgical procedure to monitor brain activity with a telemetric electrocorticogram. These mice will be kept for up to 6 months following the application of the telemetric device. Other surgical procedures (decreasing the diameter of arteries running to the brain) and hypoxic chambers will be used to reduce blood flow or oxygen to the brain of neonates or adults (to mimic early changes seen in Alzheimer's Disease). These mice may be kept for up to 9 months. We will include groups of mice with sham operations to make sure that the changes observed are due to the procedures and not due to the mice undergoing surgery. The number of mice will be limited to a small group with the power to ensure that any differences can be detected.

Modify the sleep/wake cycles: In all protocols, there is an optional step to alter the lighting to modify the sleep/wake cycles using dark cages. This will be to improve wellbeing and to understand how the circadian rhythm affects brain function. We expect the mice in altered lighting to have better wellbeing.

Pregnancy: Some mice will be encouraged to fall pregnant during remyelination after ingestion or injection of a small amount of a demyelinating substance. These mice will be kept for up to 4 months following demyelination.

Behavioural testing: there is the option to perform non-harmful behaviour testing (horizontal ladder, rotorod and field tests) to test for cognitive/motor impairment may be used. We do not expect these tests to negatively affect the rodents. Less than 20% of the mice will undergo behavioural testing.

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

General Impacts:

Most of these animals after breeding and maintenance will be killed humanely via a Schedule 1 method.

Some rodents will undergo additional procedures, with varying levels of impact.

Specific Procedures and Potential Adverse Effects:

Gene Expression Changes:



We do not expect adverse effects caused by the genetic mutations. About 80% of rodents will receive tamoxifen injections.

There may be some epileptic activity which we will monitor closely.

Potential effects include pain at injection sites, changes in appetite, weight loss or gain, and occasionally swelling of the male scrotum.

Brain Injections:

Less than 5% will have substances injected into their brains. There is a low-risk of anaesthesia-related complications, minor weight loss, and very rare infections or behavioural changes. Wound closure failure may occur rarely (<5%) but will expose the animal to temporary risk of infection and tenderness around the site.

Brain Activity Recording:

Some will have devices implanted on their heads to measure electrical activity. Possible issues of surgery include hypothermia, dehydration, dry eyes, minor surgical wound problems, and rare post- surgery infections or device displacement.

Behavioural Testing:

Up to 20% will undergo simple physical tests (e.g., running on a treadmill). To test the effectiveness of a drug, we may test the pain threshold in mice with well-known tests that are thought to be mild and transient. These tests are not expected to cause significant harm.

Physiological Sampling:

Less than 5% will have samples taken. There is potential for mild stress, minor bleeding, bruising, or very rarely nerve injury.

Epilepsy Testing:

Some will be injected to induce epilepsy. Epilepsy might cause their whole body to jerk, which indicates the whole brain is included in the increased brain activity. We carefully monitor mice and once we know that epileptic activity is occurring, the mice are humanely euthanised and the tissue is taken for analysis.

Specific Protocols and Their Effects:

Myelin Loss:

To mimic diseases where myelin is damaged in the brain, like Multiple Sclerosis and Alzheimer's Disease, we will cause myelin loss by changing the rodent diet or injecting substances that damage myelin. These may lead to weight loss and sensory or movement issues, which in protocol 7, which mimics the disease development of Multiple Sclerosis specifically, it is a severe protocol. We will score their sensations and ability to move and their welfare every day using a humane scoring system. If the rodents are displaying signs of ill health or welfare, we will immediately euthanise the animals to minimize their discomfort.

Mother-Baby Cell Transfer:

To image cell transfer in mothers and babies we will use drug-induced myelin loss in mothers before pregnancy. Potential effects include weight loss. The animal will be culled if the loss of weight is greater than 15%. The drug will be removed before the mothers are able to get pregnant and then the mothers will be allowed to get pregnant during the recovery period.



Reduced Brain Blood Flow:

Some mice will undergo surgery to apply microcoils to some large blood vessels in their necks, which reduces blood flow to the brain, with a very low risk of sepsis. Local anaesthetics and analgesics will be applied to prevent pain and sepsis. If sepsis occurs, the rodent will be humanely killed. There will be possible mild weight due to use of anaesthetics. The general health (weight and mobility) of the mice will be monitored.

Low Oxygen:

To mimic high altitude, rodents will be kept in cages kept at different oxygen concentrations. Close monitoring ensures any distress or low oxygen levels are promptly addressed. Over time, mice are expected to adapt to the hypoxic conditions. These adaptations may include changes in blood vessels to improve oxygen delivery and protective responses against certain conditions like heart attacks.

Throughout the experiment, the health of the mice is closely monitored. Daily checks are conducted to measure their blood oxygen levels. If any signs of distress or low oxygen levels are observed, appropriate measures are taken to ensure their well-being, such as providing extra oxygen.

Epilepsy During Sleep-Wake Cycles:

Brain activity will be recorded in some rodents with implanted recording devices glued to the heads of the rodents. The mice will have pain relief given before and during the surgery. Epileptogenesis may be tested in some of the mice. Humane euthanasia is provided if severe seizures occur, or last longer than a few minutes. At the end of this protocol the animals will be killed via a humane technique.

Overall:

All procedures are carefully monitored to minimize discomfort and pain. Animals showing severe adverse effects are humanely euthanized to prevent 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)?

Mice 10% mild (breeding protocol 1), 70% moderate (protocols 3-6, 8-12), 20% severe (breeding protocol 2, protocol 7).

Rats: 80% moderate (protocols 3-6, 8, 10-12), 20% severe (protocol 7).

What will happen to animals at the end of this project?

- Killed
- Used in other projects

A retrospective assessment of these predicted harms will be due by 18 December 2029

The PPL holder will be required to disclose:



- What harms were caused to the animals, how severe were those harms and how many animals were affected?

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 chosen rodents (mice and rats) because they are mammals with a close physiology to humans. They obviously lack our complete cognitive capacity, however, individual cell types work in a very similar way. We can use rodents to mimic diseases found in humans. As rodents have been used for all of these disease models for some time, including the epilepsy, Bilateral common carotid artery stenosis, and demyelinating models, and we can build upon and compare our results with those published. Where possible we validate our findings using cell cultures from human cells. However, we cannot just use cell cultures as cell lines that are supposed to mimic (say) neurons or oligodendrocytes will never recapitulate the intricate arrangement of the extracellular space that occurs in vivo. We look at young neonatal mice to understand physiology during development (e.g. pathological mechanisms activated during perinatal asphyxia), and adult mice (up to 1 year) to study pathology found in adults.

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

We already use some non-animal alternatives, including bioinformatics, human experimental/clinical data and where appropriate, in vitro and ex vivo tissue as the main foundations of our work directed at understanding and dissecting mechanisms of physiology and pathology. We use human inducible pluripotent stem cells to make cultures of neurons and glial cells in order to investigate mechanisms in a way that does not use animals. However, cells in a dish do not act in the same way as within the brain. To help with that we are sourcing human ex-vivo tissue which is available after glioma and epilepsy surgeries. We intend to validate any findings we have from rodents using this tissue. However, this will be rarely available to us. In order to understand how pathology actually develops, we will need to use the animal models.

Why were they not suitable?

The cells do not behave in the same way in vitro and change their expression of hundreds of genes that encode membrane proteins and signalling molecules (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4662426/>). Also, there is no blood supply in a dish and we therefore cannot work on the vasculature which needs blood flow to function normally.

A retrospective assessment of replacement will be due by 18 December 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your experience?

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?

Firstly, the project includes the use of multiple mouse lines that will allow for intracellular imaging of calcium or genetic knockout of proteins within specific cell types. Therefore, the majority of mice will be needed to maintain the colonies of the genetically altered mice. Many of these mice will be used for ex vivo tissue, and therefore will be culled as part of the Protocol 1 and 2 breeding protocols. We have estimated the use of 5000 mice for protocol 1 and 2 breeding.

A proportion of these mice, or wildtype mice bought in, will then be moved to protocol 3-11. In order to estimate how many mice we use in each experiment, we do calculations based on the variability of the recordings and the size of the change we are expecting to observe. At this point, these are estimates. In order to determine how many mice we will need overall, we need to also know how many experimenters are using the license, and how many experiments will be done. Although we have an idea, due to current grant funding, how many mice we will need, the funding does not cover the same period of time as the license, and we may gain further funding for more experimenters. At present, as only mice can be genetically modified, we only plan to do a smaller number of experiments with rats, that will not have any genetic modifications. Therefore, the overall numbers are much lower.

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

When possible we design experiments to use the minimum number of animals sufficient to achieve a desired level of statistical significance in the results. We will use either parametric (Student's t-test, Dunnett's test, ANOVA) or non-parametric (e.g. Mann-Whitney U-test) tests as appropriate and consult statisticians where necessary. However, we have considerable experience in this type of work, and have published extensively in peer-reviewed journals. Thus, we already have a very good working knowledge of the optimal way to design and execute these types of experiment. By using transgenic technology to make cells of a particular type fluoresce a particular colour, we can greatly reduce the number of animals used.

This is because this approach enables ready identification of cells, removing the need to record from a much larger number of unidentified cells and do post-recording antibody labelling (which often fails) in order to identify them.

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 minimise the number of animals used, we share tissue from each animal between different researchers in the lab (e.g. sharing slices from the same piece of brain, or slicing one part of the brain for one person and another part for another person). Working mainly on brain slices reduces animal use because in vivo each drug used to



manipulate signalling pathways can only be given once, while a single animal gives ~10-20 brain slices, each of which can have a single drug applied. Very early on in experiments, we will determine the variability of our control data in order to work out appropriate group numbers for statistical analysis.

Where possible we will share tissue in different sets of experiments, and use one animal for multiple experiments. For instance, when taking brain slices for patch-clamp recordings, we will take the optic nerves for analysis by immunohistochemistry or quantitative PCR.

We have a colony management system and will make sure that mouse births are planned and that each mouse is allocated in the most efficient way.

A retrospective assessment of reduction will be due by 18 December 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

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.

Firstly, the majority of our mice are from Protocol 1 and 2, and are immediately culled via a schedule 1 method to obtain tissue and will have minimal suffering.

In protocol 3, we will decapitate neonates instead of using cervical dislocation or lethal doses of anaesthetics, because this is the most humane way to get the tissue without crushing it or changing important cell signalling with anaesthetics or force.

This is very quick and minimises distress to the animals.

In Protocol 4-5, we will test the effects of gene modification or vehicle substances on cell function. This protocol is important to measure these changes and to make sure there are no unwanted side effects.

In protocol 6, we will use an appropriate concentration of cuprizone ingestion to cause demyelination in the brains without distressing the animals too much or causing severe impairments. When observing the animals it is hard to tell that these animals have demyelination, so this is tested with MRI or *ex vivo*.

In Protocol 7, we induce demyelination either by injecting substances that increase autoimmune attack of myelin, or by activating channels known to be expressed by oligodendrocytes. We will monitor the mice daily as some motor impairment is expected.



We will use the same amount of substance as has been published in order to be able to compare our results to those already produced. We hope to test therapies on these mice to decrease demyelination. If distress is caused, we will minimise this in an appropriate way.

In Protocol 8, we will induce epilepsy. For kindling, we will use the smallest dose necessary to reach our objective which is to increase propensity to seizure. A final bolus of epileptogenic drug is given, and we will give as small a dose as possible to be able to separate the effects of our control and drug groups. Where possible this will be just 40mg/kg which causes epilepsy in around 50% of wildtype mice.

In Protocol 9, we will use the lowest concentration of cuprizone necessary to cause demyelination. We will then take away the cuprizone to allow for remyelination and during this period we will allow the females to get pregnant to understand how cells from the foetus affect remyelination. We do not need to observe a big amount of demyelination to observe remyelination and therefore the time they ingest cuprizone will be less than in protocol 4.

In Protocol 10, We will reduce blood flow to the brain with microcoils which are of an optimal size to cause mild hypoperfusion. The size (between 0.16 and 0.18mm) has been tried and tested already and is known to reduce blood flow by approximately 20%. This is important to mimic hypoperfusion in humans. These mice have been kept for 9 months by other groups and therefore we do not expect serious adverse reactions during this time.

In Protocol 11, we will reduce oxygen consumption by placing mice in a hypoxic chamber (from 20- 11% oxygen), but expect the mice to compensate for the lack of oxygen inspiration by increasing their blood flow. With time we expect to see some cognitive impairment, but as with humans, we do not expect the mice to suffer as a result, and we will be monitoring oxygen concentrations regularly to make sure that blood O₂ never goes below 60%.

In protocol 12, we will implant telemetric electrocorticogram devices on mice in order to record their brain activity 24/7. Due to the surgery, there may be some discomfort and we will use appropriate antiseptics, analgesics and anaesthetics to ensure the comfort of the mice who live with the devices for up to six weeks.

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

It is important that we use an animal that is as similar to humans as possible while still being the least sentient possible. Where possible, we use immature mice, however, to mimic pathologies that occur in adulthood, like multiple sclerosis and dementia caused by hypoperfusion, it is important that we use models with adults.

This is especially important because developmental cells and aging cells do not express the same genes and proteins and function in the same way. We cannot use mice that are terminally anaesthetised because we are studying chronic pathologies where mechanisms lead to neurodegeneration over time. Also, to study brain activity, we need the mice to not be anaesthetised, as this changes brain activity.

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



Over the course of the project, we will continue to refine the protocols, for instance by introducing dark cages to maximise well-being and improve/speed recovery after surgeries. We will always make sure we use the most appropriate and up-to-date anaesthetics and analgesics to minimise pain. We will monitor the animal's welfare to make refinements if any problems arise. For cognitive assessments, which use tests that have minimal adverse effects on the rodents which include the rotorod, treadmill and horizontal ladder. We restrict their time doing these tests and habituate the animals to the environments to minimise stress.

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

There are multiple sources of published guidance for best practice, including the National Centre for Replacement, Refinement and Reduction of Animals in Research (NC3Rs; <https://www.nc3rs.org.uk/>) that we will use including the following:
Responsibility in the use of animals in bioscience research (NC3Rs) <https://www.nc3rs.org.uk/3rs-resources/responsibility-use-animals-bioscience-research>
Prescott and Lidster, 2017, Improving quality of science through better animal welfare: the NC3Rs strategy. Lab Animal 46(4):152-156 <https://pubmed.ncbi.nlm.nih.gov/28328893/>

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

I will attend events and workshops about the 3Rs, and also I am part of the local Biological Services Unit users group, which meets quarterly to discuss animal welfare and other important matters relating to the 3Rs. I also keep up-to-date by reading the 3Rs newsletters and have watched some of the webinars provided on the NC3Rs website.

A retrospective assessment of refinement will be due by 18 December 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?



20. Target investigation and characterization of potential cardiovascular therapeutics

Project duration

5 years 0 months

Project purpose

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

Key words

Cardiovascular disease, Atherosclerosis, Heart failure, Heart attack, Therapy

Animal types	Life stages
Mice	adult, aged
Rats	aged, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

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 support the investigation of potential targets for the treatment of cardiovascular diseases, and to perform an assessment of the possible effectiveness of potential medicines before animal safety tests and human clinical trials.

A retrospective assessment of these aims will be due by 18 December 2029

The PPL holder will be required to disclose:



- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

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 blood vessel diseases are still the leading cause of death and long-term disability worldwide. An area of high unmet need, cardiovascular diseases can occur throughout the lifespan, often concurrently with diabetes, obesity, chronic inflammation, and as a disease of ageing.

Historically, cardiovascular drug development has targeted mechanisms such as blood thinning, reducing cholesterol, and lowering blood pressure. If successful, novel targets and treatment approaches will improve the quality of life of patients and reduce the economic burden of disease.

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

Studies performed under this license will contribute to the understanding of the role of previously identified targets for treatment. This may be by improving confidence that medicines and other methods of altering these targets will result in a significant benefit for patients, or alternatively that they are not involved in the disease of interest so different targets should be explored.

Where there is confidence that altering a target is likely to provide patient benefit, further studies will be performed using animal models to examine the efficacy of specific potential medicines, with a view to identifying those treatments with sufficient promise to take forward into safety assessment and ultimately human clinical trials.

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

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

In the short-term, clients, project teams and funding bodies will be able to make progression decisions to better focus their limited financial resources on potential medicines with the highest likelihood of clinical success.

In the medium-term, stopping the progression of projects with little chance of success, either due to lack of target relevance or the inability to develop a potential medicine with a clear beneficial effect in animal models of cardiovascular diseases, will mean that animals will not be used in safety assessment and human volunteers will not be put at risk in clinical trials.

In the long-term, the successful identification of validated disease-modifying targets and treatments for cardiovascular diseases will result in improved clinical outcomes and quality of life for patients and carers and reduced economic burden on healthcare systems.



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

Where there may be broader interest in an animal modelling approach, and if the studies are not subject to confidentiality agreements, these will be published or shared at relevant conferences.

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

Species and numbers of animals expected to be used

- Mice: 10000
- 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.

Adult mice and rats (including genetically altered animals) will be used as these have been demonstrated to provide information that enables decisions to be made on the progression of projects. Using genetically altered animals allows the investigation of human-specific targets and treatments for these targets. Where possible, male and female animals will be included in projects to support clinical translatability.

These animal studies serve as a bridge between computer- or cell-based experiments and human clinical trials. These studies will, in many cases, be the final studies investigating the likelihood of a potential medicine having a clinical benefit before clinical trials are started.

Aged animals may occasionally be used when studies investigating diseases strongly associated with older patient groups, such as cardiac fibrosis and heart failure, are undertaken.

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

Typically, animals will be purchased from a commercial supplier, including genetically altered lines and aged animals, and will then:

Be used in procedures where blood and tissues samples are taken under general anaesthesia from which the animal does not recover. This would be a small number of animals, and the tissues would be used for cell-based and biological tests.

Be dosed with potential medicines usually orally, under the skin, or into the vein on one or more occasions, dependent on how the drug accumulates and is broken down by the body, up to 6 months dependent on disease model utilised, to investigate the action on the target system of interest. Dosing by non-standard routes, such as direct injection into the heart, may be done during surgical procedures.

Dosing of some potential medicines may be undertaken following induction of disease,



such as with dietary modification, under general anaesthesia, during surgery and/or following chemical or surgical induction of a disease model, such as a heart attack or heart failure. In addition to standard dosing routes such as subcutaneous and intravenous, dosing via non-standard routes, such as injection into the heart muscle may be required. Administration of potential medicines such as cell therapies to the site of tissue damage, as in cell therapy clinical trials.

Be administered an experimental vaccine no more than once weekly on up to 3 occasions during 24-week period, usually subcutaneous injection. Blood samples would be taken approximately weekly to assess the degree of immune response.

Dosing by standard routes would usually be no more than 4 times daily for up to 7 days but in approximately 20% of studies may be up to 6 months, for example when investigating treatments for chronic diseases.

To understand how a potential medicine affects a disease pathway, such as surgically induced heart failure, a treatment may be given prior to the surgical procedure. This may be through long-term administration (up to 6 months) if the potential medicine is a preventative therapy, or a single dose treatment during the surgical procedure of a potential medicine known to affect the pathway of interest.

These studies may include using aged animals, or genetically altered animals with relevant targets deleted or human-specific target systems inserted.

Surgical cannulation of blood vessels may be required to make administration and/or sampling less stressful for the animal than using a non-surgical approach.

The effects of the target or potential medicine will generally be assessed by looking for markers in the blood, meaning samples will be taken at intervals relevant to the disease. Blood samples taken may also have the levels of potential medicine assessed to allow the amount of effect on the tissue or target to be understood.

Non-invasive serial imaging may be performed to follow changes in function, structure, or processes such as inflammation, and the effect of the target or potential medicine. Typically, this may be up to 4 times in one day, daily over 7 days, or weekly for up to 3 months.

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

Administration of substances causes stress and pain due to handling and needle insertion. These are controlled by skilled handling and minimising the numbers of administration and sampling events.

Mild effects of short-term dietary modifications, such as high fat feeding as described in this license, will cause changes in blood markers. Effects of long-term high fat feeding may include outcomes such as obesity, heart issues, and arthritis, if fed for extended periods of time.

Some genetic alterations can cause adverse effects which are very dependent on the gene. We will primarily use genetically altered animals that have Mild adverse effects, with <10% of studies using genetically altered animals that have Moderate adverse effects.



Anaesthesia for surgery or imaging can result in heat loss and unpleasant experiences when recovering. Animals will be closely monitored, have heat supplied throughout the surgical or imaging session and only undergo the minimum number of anaesthetics required to give a satisfactory scientific output. Single housing will be used only where required for post-operative recovery (Protocol 3), or upon welfare or veterinary advice with the agreement of the NACWO/NVS.

Surgery on the heart or blood vessels in the chest of mice and rats increases severity to Moderate. Surgery will cause pain that is controlled with pain-relieving drugs, and can cause weight loss, abnormal behaviours such as isolation, and reduced food and water intake. These adverse effects can last usually up to 72 hours.

Most animals undergoing surgical procedures (~85%) reach Moderate severity, with up to ~10% of animals euthanised under anaesthesia during surgery, or within the first 24 hours post-surgery (up to 5%) due to acute clinical indications. Outcomes can usually be predicted based on early clinical indications during recovery in the first hours after surgery, with animals euthanised before Moderate severity endpoints are reached.

Animals are closely monitored and provided pain relief, fluid replacement, palatable foods, and maintained in a warming chamber to support recovery following surgery. In all surgical models, suffering will be minimised with the above interventions and conscientious husbandry post-surgery, such as during observation, compound administration, and weighing. In surgical models of heart injury, adverse effects that may be present in the first 24-72 hours post-surgery can include lethargy, respiratory impairment, acute weight loss, dehydration, difficulty maintaining body temperature (particularly in nude rodents), and reduced appetite. Adverse effects may also be present due to model progression. As cardiac surgical models emulate the disease pathology seen in humans, adverse effects may be Severe.

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)?

It is expected that animals undergoing the administration of substances and the effects of dietary modifications such as high fat feeding will experience Mild severity.

Surgery on the heart or blood vessels in the chest of mice and rats increases severity to Moderate. In surgical models of heart injury outcomes such as respiratory impairment, failure to return to pre-surgery weight usually within 72 hours after surgery, gradual weight loss of <20% within the first 7 days after surgery, or significant functional impairment as measured by non-invasive imaging, usually 7 days after surgery, are closely monitored.

Based on previous experience, most mice and rats undergoing cardiac surgical modelling procedures do not exceed Moderate severity (~85%), with up to ~10% of animals euthanised under anaesthesia during surgery, or within the first 24 hours post-surgery (up to 5%) due to acute clinical indications before Moderate severity endpoints are reached. Sudden death may occur as an adverse effect of surgical procedures (Severe severity). Based on previous experience this occurs in <1% of animals. However, this can occur in >1% of animals and is dependent on the strain, genetic modifications, sex, and age of the animals used, and the expertise and experience of the technical team conducting the



procedures.

What will happen to animals at the end of this project?

- Killed

A retrospective assessment of these predicted harms will be due by 18 December 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

Replacement

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

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

Many of the biology studies performed to help in the validation of targets and assessment of potential medicines now take place in computer, genomic, receptor and cell-based experiments (as well as definitively in clinical trials in the patient population). Integrated cell-based systems (the "organ on a chip" or even "human on a chip") are improving year on year and replacing animal usage.

However, even with these advances, computer and cell-based approaches do not allow the response of the target or potential medicine to be assessed in a way that reflects the full complexity of an integrated biological organism. This project aims to provide the data from a complex, integrated organism to allow decisions to be made on whether to progress targets or potential medicines to the next stage of development.

The use of non-mammalian species is not a relevant approach due to the degree of differences at a genetic and molecular level in these models. They are more suited to very early scientific investigations rather than drug discovery and development.

In the heart, repair and remodelling requires blood to be pumped through blood vessels. While some elements can be modelled outside of the body, such as the formation of new blood vessels, it is not possible to reproduce the force produced by the heart when pumping, or the complex repair processes occurring after injury.

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

Some or all computer modelling, genomic, cell-based and non-protected animal approaches will have been used prior to undertaking studies on protected animals to minimise the number of studies and impact on the animals.

Clients will be asked to provide information on the work undertaken with approaches not using protected animals and an outline of literature reviewed searching for alternative approaches prior to performing in vivo studies. It would be expected that in vitro binding, cell-based biomarker assays and where possible human tissue studies will have been undertaken.



These studies will usually include ability to affect the target, assessing the properties of potential medicines for biological availability, likelihood of reaching tissues of interest, and looking for early indications of toxicity liabilities. These tests will reduce the numbers of experiments performed and increase the likelihood of those completed to deliver meaningful results.

Why were they not suitable?

There are no non-animal alternatives that can currently replicate the full complexity of the mammalian body. Now and for the foreseeable future there will need to be animal experiments performed to investigate target validity and the efficacy of potential medicines as part of the process of bridging from non-animal studies to clinical trials.

A retrospective assessment of replacement will be due by 18 December 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your experience?

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 estimates are based on the number of projects we anticipate supporting, with typical studies comparing genetically altered and/or treated animals with control animals. We estimate that we will be supporting the in vivo work for in the region of 5 client drug discovery programmes, plus fee for service stand-alone studies at any time under the authority of this licence.

All new models and major changes to established models requiring amendment will undergo review by AWERB. Named Person's advice that impacts animal numbers without reducing scientific validity is received on an ongoing basis and implemented where relevant.

Most studies will be carried out using mice, but rats may be preferred in some instances. Based on the literature, a typical study may use up to 15 animals per group and up to 8 groups, including positive and untreated control groups, and multiple compounds at different dose levels. Running fewer, larger studies will reduce the number of positive and negative control animals used, whilst providing greater confidence in the results.

Studies will be designed to gain as much information as possible from each animal without compromising welfare. For example, in long-term studies it is possible to assess mechanistic and functional changes throughout the study with imaging and via biomarker assessment, without euthanising the animal until the defined terminal timepoint. Well-characterised methods established in the literature, and replicable protocols, will ensure



comparability between operators and studies.

Additionally, we anticipate providing tissues from genetically altered animals for cell-based research, such as where these are not available from other commercial sources at a suitable quality. The estimate is for approximately 6 animals per week to be used for this purpose.

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

We will use pre-existing data, including published and internal pilot data, to perform power calculations that ensure group size is likely to provide a robust experimental output without using excess animals.

This will usually be with the support of a biostatistician, but if this is not possible power calculations will normally be performed.

In rare cases where there is no initial data to guide power calculations the Mead Resource Equation will be used to estimate sample size

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

Wherever possible pilot studies will be performed to help design the decision-making studies. If studies have been performed on how the potential medicine is absorbed and removed from the body, modelling will be used to predict the doses required for these studies. Using these pilot studies to understand how the experimental measures vary will also help design the decision-making studies.

Review of model performance and of standard protocols that are run repeatedly will ensure that they continue to operate optimally. This will be performed routinely, such as after each time a model is repeated at regular intervals.

In some studies, especially those with a higher risk of adverse events, tissue samples will be taken after study completion and may help prevent further studies being performed should unexpected results be found.

When providing tissues and blood an email distribution list will be used to allow sharing of tissue throughout the company for use in cell-based studies, minimising the overall number of animals required.

A retrospective assessment of reduction will be due by 18 December 2029

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

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

Rodent models of cardiovascular disease are reproducible and suitable for clinically relevant modelling of human disease. A breadth of published data is available and protocols for pathophysiologic disease induction well-established. Genetically altered animals with global or inducible gene modifications that demonstrate no, or Mild signs, will be used where suitable. Suitable models with Moderate severity will be chosen when relevant to the target, where required these will be obtained from commercial suppliers and/or research establishments.

Key elements required for models:

Provides a measurable biomarker of disease progression

Is conducted for a minimum duration to provide meaningful data

Analgesia is used where pain could occur and in agreement with the NVS

Continual assessment of the literature and published methods to ensure the latest refinements are employed relevant to the disease of interest

Includes clinical support where indicated, such as placing animals into a warming chamber, providing fluid and dietary support when clinical monitoring indicates weight loss (<20%), and using enriched oxygen during thoracic surgical procedures

The use of temporary blood vessel cannulation, where appropriate

The overall premise of the license is that the most refined, most relevant and least invasive method will be used at each stage for the administration of any substances and sampling procedures. When the experimental question justifies additional burden to the animal (e.g., surgery, single housing, or chronic administration) we will aim to keep such burden to minimum levels:

The administration of substances under this licence will be with the smallest volumes commensurate with the aims of the procedure.

The use of either direct venepuncture, temporary or surgical cannulation for sampling will be based on determining factors such i) the robustness and accuracy of sampling necessary, ii) the study length, iii) the frequency and volumes of samples to be collected, and iii) to minimise burden on the animals during sampling.

Surgical techniques and post-operative recovery standards will be continuously monitored and revised. Surgical procedures, including preparation, surgery and recovery care, will be performed in keeping with current best practice guidelines (LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery). Peri-operative analgesia will also be used and maintained for as long as necessary in accordance with our experience in using the models as requested on this licence with the use of grimace scale to monitor pain in the animals.

Where possible, we will explore alternatives to single housing but when this is not possible, such as with post-surgical animals and those with surgically implanted cannula, they will be singly housed with selected enrichment where the loss or damage to the cannula can



be managed.

On chronic studies, the dose route will be carefully discussed with the client and alternatives to repeated injections, such as implantation of minipumps, will be contemplated.

For surgical procedures, the most refined and minimally invasive procedures will be used, for example utilising JVC for serial blood collection, a refined and robust procedure that has been established as the standard for taking accurate repeat blood samples from the same animal in longer studies with higher sampling frequencies. Additionally, both the route of administration and sampling regimens will follow refined best practise, such as: Compliance with LASA guidelines (Good Practice Guidelines - Administration of Substances)

Administration of Substances to Laboratory Animals: Routes of Administration and Factors to Consider, Turner et al (2011)

A good practice guide to the administration of substances and removal of blood, including routes and volumes, Diehl et al (2001)

Surgery carried out in-house

Animals will be given peri-operative local and systemic analgesia such as carprofen which will be re-administered according to best practice guidelines. Animals will be monitored frequently & regularly for comfort following recovery of anaesthesia for signs of pain using both grimace scales and general observations of animal movement. After the initial 24h period animals will be reassessed to determine if they are in pain following withdrawal of analgesia, additional analgesia will be administered as required.

Models where a pathway of disease interest is stimulated (e.g., by surgical induction of heart failure, or a genetically altered or aged animal that develops heart failure) will be chosen to investigate whether a target is significantly involved in, or a potential medicine affects, that pathway.

Coronary artery ligation (CAL) which restricts blood flow to the heart muscle is the established model to induce heart failure (HF). Brief, temporary coronary artery occlusion which restricts blood flow for a short period of time before allowing normal blood flow, such as occurs during a heart attack with interrupted blood supply, is the established model to induce ischaemia-reperfusion injury (IRI).

Outcomes can be predicted based on recovery in the first hours after surgery, and animals are euthanised before severity endpoints are reached.

Based on previous experience, most animals undergoing surgical procedures do not exceed Moderate (80-85%), with up to 15-20% of animals euthanised during surgery or within the first 24-72 hours post-surgery. Like humans, uncommon, sudden deaths may occur as an adverse effect of heart failure surgery. Surgery will cause pain that is controlled with pain-relieving drugs, the animals are closely monitored and provided pain relief, fluid replacement, palatable foods, and maintenance in a warming chamber to support recovery following surgery. In all surgical models, suffering will be minimised with the above interventions and conscientious husbandry post-surgery, such as during observation and weighing.

Where suitable non-recovery surgical models will be used. For example, acute studies requiring induction of IRI and immediate treatment to assess therapeutic mechanisms, or direct cardiac muscle injection of a therapy following CAL. If vaccination is required, these



will be managed with pain-relieving drugs to reduce any adverse effects that may occur.

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

The use of non-mammalian species is not a relevant approach due to the degree of differences at a genetic and molecular level in these models. Due to the regenerative capacity of heart cells in less sentient models such as zebrafish, and in neonatal rodents, CAL results in cardiac regeneration rather than the development of a disease state and as such, they are not suitable for the induction of HF. They are more suited to very early scientific investigations rather than drug discovery and development.

In the heart, repair and remodelling requires blood to be pumped through blood vessels. While some elements can be modelled outside of the body, such as the formation of new blood vessels, it is not possible to reproduce the force produced by the heart when pumping, or the complex repair processes occurring after injury.

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

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

Scoring systems are used to identify early intervention and end points in studies, pain-relieving drugs are used when there is concern an animal is suffering, and monitoring is performed as often as required, including throughout the night.

Further refinement to technical procedures and housing are implemented when they are shown to be beneficial for the animals and will not interfere greatly with performing the studies.

When an established model is identified from the scientific literature and proposed for use under this license, a small number of pilot studies will be performed to ensure the model delivers the expected changes in the pathway or tissue of interest. These pilot studies will also be used to determine the controls that may be used to reduce the severity experienced by the animals and the study duration. An iterative review process will be conducted to assess model performance, to maximise scientific output and minimise animal suffering.

We have multiple examples where protocols have been adapted based on observations in studies, in many cases the changes are modest, such as modifying administration route to less painful injection sites, reducing dosing volumes, or using non-invasive monitoring to assess clinical deterioration.

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

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

How will you stay informed about advances in the 3Rs, and implement these



advances effectively, during the project?

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

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

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

A retrospective assessment of refinement will be due by 18 December 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?



21. Toxicology Testing of Chemicals

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

Environmental, Toxicology

Animal types	Life stages
Sheepshead Minnow (Cyprinodon variegatus)	juvenile, embryo, neonate
Zebra fish (Danio rerio)	juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

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 provide information on how toxic a chemical is to fish. The chemicals tested will mainly be used in the oil and gas industry. The information gathered will then be used by regulators to decide if the chemical can be used in the industry and what controls (if any) will be placed on the chemicals use.

A retrospective assessment of these aims will be due by 17 December 2029

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?



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 testing allows the regulator to know how harmful the chemical is and allows them to prevent or restrict the use of anything that is likely to have very negative effects on the natural environment.

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

The testing will let our clients know at which concentration their chemical can be considered not toxic to the fish. Alternatively, they will get information on which concentration of their chemical will kill 50 % of the fish exposed.

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

The data gathered is submitted by our clients to regulatory bodies controlling the use of chemicals in marine and freshwater environments.

The use of animals in a laboratory setting is expected to minimise the impacts caused to wildlife by stopping the use and/or release of toxic chemicals. The data gathered in these tests is used by the regulator to determine if any controls need to be placed on the substance's use or if it needs to be switched to something that is less harmful, thereby protecting fish (and other marine life) populations in the wild. In combination with algae and crustacean testing, it provides a better understanding of the overall picture of the effects on the marine environment.

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

Work is confidential to each client.

Species and numbers of animals expected to be used.

- Other fish: No answer provided
- Zebra fish (*Danio rerio*): 100

Predicted harms

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

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

The guidelines for the registration of chemicals used in the offshore oil and gas industry say that fish testing must be conducted on the chemicals to be used on oilfield sites. It is not possible to replace this stage of testing with an alternative. Algal and crustacean tests are also conducted on the chemicals but are in addition to the fish testing. All results are needed to register the chemical.



After a course on the future of fish toxicity testing attended on 28 June 2022, all licence holders have been aware of the work being done involving the use of fish gill cells to determine toxicity in the animals. This is not yet an established method and is not accepted by regulators, so it is not possible to replace the full animal yet, however, ongoing monitoring of that test is regularly done with the view of replacement.

Clients are encouraged to use the OECD 210 testing as it uses animals that are not yet protected, however, it is not always possible for regulatory purposes.

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

For acute/limit testing, fish will be added to water containing the chemical via nets. Generally, a single concentration of the chemical will be used per test, which is determined to be suitable from previous bench-top tests. Experiments will last no longer than 96 hours and will be stopped earlier if there is deemed to be any excess suffering of the fish. Fish are killed at the end of the test.

An estimate of 6000 marine fish over 5 years will be used in acute/limit testing.

Sheepshead minnows are the main marine fish used under the project licence. For freshwater acute/limit testing, around 100 zebrafish may be used. Around 100 turbot may also be used for acute/limit testing. No zebrafish or turbot were used under the last project licence.

For egg chronic tests, eggs will be exposed to known concentrations of a test material.

The test duration is dependent on the species (for sheepshead minnows it is approximately 32 days). Hatching and fish are observed daily. Where deformities and associated abnormal behaviour are considered so severe that there is considerable suffering to the fish, and it has reached a point beyond which it will not recover, it will be removed from the test. All fish are killed at the end of the test.

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

Although steps are taken to minimise the likelihood of severe suffering to the fish, it is not known exactly how the chemical will react with them until they are exposed.

Therefore, the fish could suffer toxic effects from exposure to the chemicals, up to and including death. Death caused by the chemical will be avoided as far as possible and will be replaced with an earlier, more humane endpoint if suffering is 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)?

The expected level of severity is mild, however, severe is possible and will be the limit.

Data from the previous PPL suggests approximately 1 fish will experience severe suffering to every 10 fish that experience mild suffering. Data since 2020 places the number of fish



experiencing the severe limit at 12.7 %, although suffering has reduced since new fish have been added to the gene pool starting in mid-2021 (2022 had 8.5 % of the fish experience the severe limit, and 2023 % had 7.9 % experience the severe limit).

It is expected that similar proportions of fish will experience these severities under this licence (for all species and testing).

What will happen to animals at the end of this project?

- Killed

A retrospective assessment of these predicted harms will be due by 17 December 2029

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

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 guidelines for the registration of chemicals used in the offshore oil and gas industry say that testing must be conducted on the chemicals to be used on oilfield sites, using adult fish. It is not possible to replace this stage of testing with an alternative. Algal and crustacean tests are also conducted on the chemicals but are in addition to the fish testing. All results are needed to register the chemical.

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

After a course on the future of fish toxicity testing attended on 28 June 2022, all licence holders have been aware of the work being done involving the use of fish gill cells to determine toxicity in the animals.

Why were they not suitable?

This is not yet an established method and is not accepted by regulators, so it is not possible to replace the full animal yet, however, ongoing monitoring of that test is regularly done with the view of replacement.

The document "*Criteria for accepting alternative toxicity data to support an HOCNF application*" downloadable file from CEFAS gives the criteria that data must meet in order for it to be acceptable under the Offshore Chemical Notification Scheme. This document lists the OSPAR fish testing as a necessary component for registration.

The HOCNF form used by clients for submitting their chemical includes the required section for a fish test result.



A retrospective assessment of replacement will be due by 17 December 2029

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your experience?

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 estimated from the total number of animals used under previous project licences for this establishment.

Usually 10 fish are used per test. 2022 and 2023 saw 85 tests each which is expected to continue through this licence, therefore 4250 sheepshead minnow are expected to be used. Extra numbers are added to accommodate an increase in test work and the potential increase from egg chronic testing, which is expected to begin during the course of this licence.

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

For standard testing, only one test concentration is used for the chemical. This concentration is based on the results of the algae and crustacean tests, and the fish test toxicity level is reported as a greater- than (>) result. By using one test concentration, the number of fish used is significantly reduced when compared to a full range of concentrations (10 fish per test rather than up to 70 per test). All clients are encouraged to use this single concentration test and it is accepted by the regulator. When possible, tests are run in sets of at least 3 to reduce the number of control fish used throughout the project.

Sometimes less tests will be run to stop fish from growing over the maximum testing size and not being used at all.

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

When possible, tests are run in sets of at least 3 to reduce the number of control fish used throughout the project. Sometimes less tests will be run to stop fish from growing over the maximum testing size and not being used at all.

A retrospective assessment of reduction will be due by 17 December 2029

The PPL holder will be required to disclose:



- **How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?**

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.

Testing follows the guidelines to ensure the results will be accepted by the regulator. Fish that show signs of suffering above what the personal license holders consider 'mild' will be euthanised to stop suffering. If a chemical is showing significant effects on the test fish, the test will be stopped immediately, and the fish are euthanised to stop suffering. A welfare assessment chart is in place to standardise the observations and allow staff to make informed decisions about the severity level a fish is experiencing.

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

The guidelines for the registration of chemicals used in the offshore oil and gas industry say that fish testing must be conducted on the chemicals to be used on oilfield sites. It is not possible to replace this stage of testing with an alternative. Algal and crustacean tests are also conducted on the chemicals but are in addition to the fish testing. All results are needed to register the chemical.

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

Fish are monitored at least 4 times during the working day. Any fish showing potential signs of pain will be monitored more frequently to make sure suffering does not go beyond what is necessary and they can be euthanised to prevent further pain. Euthanasia will occur before the end of the working day, and no fish will be left overnight if there are any concerns for its welfare. For testing to guidelines, it is not possible to use pain management as they may interfere with the test substance.

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

We are tied to the guidelines for how refined the test work can be. We have a copy of the PREPARE checklist for use if required.

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

Client liaison staff, as well as the named information officer (responsible for gathering information on the testing and welfare of animals), frequently attend seminars where they are able to keep informed of any advancements or potential updates to the guidelines. This



information is passed through to staff who work with the fish on a daily basis. We also keep up-to-date on courses and seminars that can be attended online.

A retrospective assessment of refinement will be due by 17 December 2029

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?