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NON-TECHNICAL SUMMARY

Molecular mechanisms and new treatments for cardiovascular diseases

Project duration

5 years 0 months

Project purpose

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

Key words

myocardial infarction, regenerative medicine, aortic aneurysm, Marfan syndrome, therapeutics

Animal types	Life stages
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult, Aged animal
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?

Overall aim: To identify the causes of cardiovascular diseases (aneurysms, strokes and heart attacks) and to test new treatments for these including the use of human pluripotent stem cells (hPSCs).

A retrospective assessment of these aims will be due by 6 October 2031

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?

Cardiovascular diseases, affecting the heart and blood vessels, are the leading cause of illness and death in the developed world.

The most common problem is a heart attack (myocardial infarction), which happens when the coronary arteries, the vessels supplying blood and oxygen to the heart, become blocked. This damages the heart muscle, often leading to long-term heart failure.

Large blood vessels such as the aorta can also be affected. In conditions like Marfan syndrome, the aorta expands like a balloon (an aneurysm) and may tear open, often causing sudden death. Some cardiovascular diseases, like Marfan syndrome, are inherited, while others arise from smoking, high cholesterol, or high blood pressure.

A common feature is that damage is usually irreversible: after a heart attack the muscle cannot repair itself, and with aneurysms the aorta cannot heal without major surgery. These treatments are risky,

costly, and not always available.

Our research focuses on understanding these diseases and developing new treatments that are safer, more effective, and more widely accessible.

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

This project will generate new knowledge about how the heart and blood vessels develop, function, and become diseased. A key goal is to refine a cardiac patch and cell therapy to repair hearts damaged by heart attacks. During this licence period, we will improve this approach and assess its safety and effectiveness. In the longer term, these therapies could progress to clinical trials, offering a completely new treatment for heart failure - a major global health burden - and may also lead to patents.

We will also gain detailed insights into the molecular features of vascular cells, helping us understand how blood vessels form and how they contribute to diseases such as aneurysms and Marfan syndrome. This knowledge will guide the development of new drug treatments, which we aim to test in future clinical trials.

Finally, we will publish our results in open access journals, ensuring that scientists worldwide can benefit from and build on our findings.

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

Our research has both short- and long-term benefits. In the short term, findings will support members of our group, collaborators, and the wider scientific community through publications, shared data, and new funding opportunities. In the longer term, our therapeutic strategies for heart attack and vascular disease aim to reach Phase 1 clinical trials within the decade, with the ultimate goal of new treatments tailored to individual patients. These studies have already produced high-quality publications and will continue to advance global efforts against cardiovascular disease.

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

We will maximise the outputs of our work by presenting widely and forging collaborations - for example, we will work with groups with similar interests to share data and information. We will also make our new knowledge widely available through our website where data analysis portals will allow all researchers to access and mine our data and by publishing in open access journals. We will also share tissues or organs from our animal models that we do not use with other groups who may be interested, thereby maximising the use of our animals within the scientific community. All animal tissues and RNA (Ribo Nucleic Acid – messenger molecules transcribing the genetic DNA code to enable protein production.) or protein samples obtained will be stored and banked and made available for new studies or other researchers, to eliminate the use of additional in vivo experiments where possible. For example, if a new gene is discovered that may affect the response to cardiovascular injury, then stored samples of RNA or tissue sections could be probed to understand what happens to this gene following injury without carrying out any further in vivo studies.

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Even when approaches do not succeed, we will make tissues and data available to collaborators, consistent with the principles of the 3Rs (Replacement, Reduction, Refinement).

Species and numbers of animals expected to be used

- Mice: 12125
- Rats: 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.

We will use mice and rats, as they are well-established models for cardiovascular disease and regeneration. Mice are highly suitable for genetic modification and modelling vascular diseases such as aneurysm and atherosclerosis. Importantly, mouse studies have been successfully translated to human trials in previous work and is therefore a key model organism for treatment development and testing. Rats are essential for our regeneration studies, as human cardiovascular cells survive and function better in rat hearts than in mice, likely due to heart rate differences and immune compatibility. Other species (e.g. rabbits, pigs) are less practical due to slower breeding and limited availability.

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

Most animals will be held only up to a maximum of 15 months of age and the majority of animals on this licence will be under mild or moderate protocols, and suffer only minimal adverse effects. A number of animals will be subjected to mating/breeding procedures. Under experimental settings, several test and appropriate control animals may undergo some procedures for specified number of times. These include blood pressure measurements (animals will be restrained in a small tube-like enclosure with a tail cuff after gentle and consistent training), one of two different types of imaging such echocardiography (using ultrasound imaging) or magnetic resonance imaging (MRIs) to examine the functioning of the heart and the blood vessels. In several experiments, we will administer substances that can modify genes (e.g. tamoxifen), cause aneurysms or perform drug treatments. These will be administered via a variety of routes including - injections: intravenous (through a vein) and intra-peritoneal (into the peritoneal cavity which is the space between the abdomen and the pelvis that contains the abdominal organs), oral: such as gavage (via a tube leading down the throat into the stomach - for short-term administration only) or in food or water (for long-term administration). In some experiments, we will administer general anaesthesia, usually inhaled and/or via injections. In some cases, we will conduct blood sampling (typically blood is drawn via the vein in the animal’s tail), often for the purposes of testing drug delivery.

For specialized studies to develop treatments for heart attacks, blood vessel injury and aortic aneurysms, we need to use some animals that also suffer a heart attack, vessel injury or aortic aneurysm. These are serious conditions and frequently lead to death in patients, so these animal

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protocols are often severe in category. To model these conditions, we will induce them in the animals via different methods. For heart attacks, we will conduct open chest surgery under anesthesia (rats and mice - heart attack via closing off or constricting the coronary artery). For blood vessel injury, we will surgically (under anesthesia) constrict (blocking or closing off) a carotid artery (mice). For inducing blood vessel dilation i.e. aneurysms, we will often use mice that are genetically modified or administer drugs such as angiotensin or inject lentiviruses into the veins to only modify cells in the blood vessels (mice). These will cause the vessels to balloon and expand as seen in aneurysms. In rare experiments, where we need animals with suppressed immune reactions, we may use irradiation to deplete the bone marrow of immune cells and transplant them with genetically altered or normal bone marrow. Alternatively, we may use genetically immunodeficient (where the immune system of the animals is genetically compromised) strains for such experiments.

In typical experiments, animals will be allowed to recover after these procedures (both surgical and non-surgical) and will be monitored over time with or without specific drugs treatments and therapies. Some of these therapies may be administered to the animals during or immediately after surgery (for example, AAVs (Adeno Associated Viruses) may be injected into the heart muscle after heart attack surgery to genetically modify molecular pathways that will be affected in heart attacks). We will monitor them using a variety of methods including imaging (as described above) to assess the functioning of the heart or the condition of the altered blood vessel. This will allow us track how well the treatments are working over time to alleviate the heart attack or vessel injury or aneurysm. This will then allow us to potentially use these interventions as treatment options for humans with these diseases. At the end of the experiments, animals will be humanely killed (unless clinical signs prompt us to do so earlier) and we will utilise their tissues to thoroughly study the effects of the various treatments.

A small proportion of animals will die during the protocol. In most of these animals, death usually occurs suddenly with only transient suffering. If the animals are suffering, they will be given suitable treatment such as analgesia and if this does not alleviate the suffering promptly, they will be killed humanely. All animals will be killed humanely at the end of the studies. The enormous burden and severity of heart attacks and aneurysms in patients worldwide warrants the use of severe category animal protocols in order to find new treatments.

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

Most of the animals in our studies will at the most, only experience moderate and even if so, only transient discomfort arising from the aftermath of certain procedures such as surgeries or some injections such as pain or short-term recoverable weight loss. We have some protocols classified as severe to account for either specific strains or invasive cardiac surgery or aneurysms. These animals may experience sudden death and we have humane endpoints instituted to kill the animals at any clinical sign of prolonged distress.

One of the most severe procedures in this project is open-chest surgery in rats to conduct myocardial infarction (heart attack). Although, in this procedure a heart attack is induced with localised damage or injury to the heart muscle, we expect the heart to still function i.e. compensated heart failure. Therefore, if the procedure is successful, the animals should not show symptoms and are characteristically well and move freely in the early stages. However, if decompensation i.e. loss of heart function occurs, then there will be clear symptoms including cyanosis (blue coloration of extremities such as toes), oedema (puffy appearance due to fluid accumulation) of the extremities and

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laboured breathing. Regular observation of the animals allows these symptoms to be used as humane endpoints with immediate humane killing. As with humans, some deaths are sudden and presumed to be arrhythmic (due to irregular heart beats).

From the surgery itself, animals may experience some clinical signs like pain or weight loss. We have methods to mitigate these, for example, via modified feeding with mash and supplements or the administration of pain medication both before (preventive) and after surgery. If there is no relief or weight gain within 48 hours, we will humanely kill the animal. Some animals may experience respiratory distress, although this is rare. In cases like that, we will humanely kill the animals.

Some procedures (e.g. aneurysm formation, coronary artery ligation) may result in death in a proportion of animals (5-10% for coronary ligation i.e. myocardial infarction) in rats, 10-20% for ligation in mice and 50-80% for the severe aortic aneurysm models), and they therefore have a severe severity classification. These models are used to find targets or test treatments that reduce the formation and rupture of aneurysms or affect the response of the heart and its circulation to myocardial infarction. A severe limit may be needed where the control group needs to experience a sufficient percentage of events (e.g. rupture of aneurysms which is when the artery is highly enlarged and the thin walls break) that can be reduced by a treatment, with adequate statistical power (a sufficiently sized experimental group to allow for clear observation of patterns e.g. response to therapies after myocardial infarction) for a definitive conclusion. In our collaborator’s large previous experience with aneurysm rupture studies, 90% of deaths are sudden and could not be expected despite close monitoring twice daily. In 10% of deaths, mortality was preceded in the previous 18 hours by some signs of reduced activity or hunching. However, such signs are not specific, nor predictive of rupture because most animals with such signs recover and do not undergo aneurysm rupture. Nevertheless, if animals show signs of distress, we will monitor them more closely to try to predict those who will ultimately undergo aneurysm rupture. We will also consider doing more non-invasive imaging on those mice (ultrasound) to try to detect imminent signs of aortic rupture. In our experience, this is a highly effective and successful method to detect aneurysms and estimate when they are close to rupturing. This is because we can track the progress of the aneurysm and when the size of the blood vessel reaches a certain predetermined point (a parameter that we have carefully characterized on some of the most severe aneurysm mouse models), we will stop the experiments and humanely kill the animal before the rupture occurs.

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

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

Mice:

Mild	70%
Moderate	20%
Severe	10%

Rats:

Mild	5%
Moderate	30%
Severe	55%
Non recovery	10%

What will happen to animals used in this project?

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

A retrospective assessment of these predicted harms will be due by 6 October 2031

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?

Cardiovascular development and disease involve highly complex interactions between cells, their supporting matrix, growth factors, and blood flow. These processes cannot be fully recreated in a dish or on a computer. Studying them in living systems is therefore essential to understand how they are regulated and to design and test new therapies. Conditions such as heart attack and aortic aneurysm are especially complex, and no alternative methods currently exist that can fully replace animal studies in these areas.

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

We have made extensive use of in vitro (cell culture based) techniques to replace many of the initial studies and experiments and hiPSCs (human induced pluripotent stem cells) and hESCs (human embryonic stem cells) to model cardiovascular development and disease that can replace animal studies in part.

These “disease in dish” models where we use the hESCs or hIPSCs made from the patients themselves, allow us to successfully derive the different cell types that make up the wall of the blood vessels and the heart. The cells then recapture the features of the diseased blood vessels and failed hearts and we use them to 1. Understand how the disease works and all the molecular processes that are disrupted which eventually cause the condition and 2. To test different novel drugs and treatments

that can reverse the features of the diseased cells back to those displayed by cells from a normal person. These cell culture-based models are incredibly useful in predicting disease progression as well. Some of our methods are as follows:

1. We have established methods to generate vascular smooth muscle cells (layer of cells in blood vessels that is critical for blood flow and blood pressure and which become diseased in conditions like aneurysms and atherosclerosis) and epicardium (layer of cells surrounding the heart that are necessary for regenerating the heart tissue when injured) from human embryonic stem cells (hESCs) and hIPSCs. These techniques are able to identify key molecular mechanisms in vitro. In addition, we can build tissue engineered vessels in vitro to simulate an intact blood vessel with pressure and flow. Molecular mechanisms identified using these approaches will finally be tested in vivo using rodent models.

2. We have built engineered heart tissues using cardiovascular cells from hESCs that can spontaneously contract just like heart muscle. We can measure how much force these cells generate in comparison with a beating heart, how they use calcium to perform this contraction and estimate how mature the engineered heart tissue is in comparison with an adult human heart. These will help us understand how well these in vitro models can mimic a heart and will determine how effectively we can use the cardiovascular cells we make from hESCs. Once these have been optimised in vitro however, our findings will need to be validated in vivo.

3. We have developed methods to use human induced pluripotent stem cells (hiPSCs) to establish cardiovascular genetic diseases ‘in-a-dish’. We have recently published a model of the aortic aneurysm in Marfan syndrome in vitro and identified new disease-causing pathways. This complex in vitro model is also being used to screen for drugs to treat aortic aneurysms. Ultimately however, the pathways and drugs need to be validated in vivo.

4. In some studies we plan to use or have been using human cardiovascular tissues (taken from patients after surgery or human fetal samples after termination of pregnancy – with suitable ethical approval) to validate our hESC-derived cell models with instead of using animal tissues. In addition to comparing human to human, this approach also replaces the need to kill and use animals to provide suitable comparator tissues.

5. Finally, as an alternative to using animal models of aortic aneurysms we have collaborated with vascular surgeons and radiologists to study patient-derived CT scans (Computed Tomography scans – used to image various internal organs to determine the impact of disease or injury. In this context, we will use images of the patient arteries, especially the aorta vessel arising from the heart to determine how much disease-related enlargement is seen) to try and predict patient outcomes.

With these replacements, we have thus far been successful in replacing up to 50% of our need for animal-based studies.

Why were they not suitable?

Once we have candidate drugs or treatment strategies, we still need to test them in mice and rats first before we apply them to human patients. This is because, although the disease in dish models are ethical and highly efficient, they cannot tell us how well the drugs will perform in the organism (or

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patient). *In vitro* studies are limited in their use because cells are grown on artificial substrates and studied in isolation from the rest of the organ and from the whole body. These models can sometimes be tailored to mimic *in vivo* conditions but this is only to a small degree. Hence, they cannot determine the effects of the drug on a whole tissue or even other organs in the body. This is critical, especially when applied to human patients; for example, a drug that is effective in cultured cells, may be detrimental in the patient because of side effects on other organs.

Hence to determine fully, the therapeutic potential of drugs or the function of critical genes and biological pathways, in cardiovascular disease, we will have to use *in vivo* experiments. For this, we use nuanced genetically modified mice that will capture the disease features and simulate the conditions in patients. Once the drugs show efficacy in the mice, we will move them to clinical trials.

A retrospective assessment of replacement will be due by 6 October 2031

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 rigorous statistical methods based on information and our extensive experience from previous in vivo and in vitro experiments to estimate the number of animals we need in each experiment. These methods allow us to use only the required number of animals.

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

The experiments have been designed with several points in mind to minimise animal use and animal suffering.

First, the extensive in vitro models using human stem cells described under Replacement have enabled us to refine our hypotheses regarding underlying molecular mechanisms, thus minimising the questions that need to be asked in the animal studies as well as maximising the information obtained.

Second, archived tissues as described under ‘Replacement’ will also be used to carry out pilot hypothesis generating studies, so reducing the number of new animal experiments required.

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Third, previous studies by our collaborators on the rat and mouse models of myocardial infarction and regeneration and aortic aneurysm development have already identified the most appropriate rodent strains, the techniques required for consistent results and the time points at which the models provide the most information.

Fourth, the use of ultrasound imaging (ultrasound guided imaging of the heart and blood vessels to determine size and structure of the organ wall, its working and blood flow) of ventricular (the lower chambers of the heart –ventricles – which push the blood by contracting every cycle/beat into the atria or upper chambers) function and aortic size (width of the aorta blood vessel) as well as histological (tissue slices stained for visualizing various components of the cells and tissue) data will allow us to increase the amount of information we get from any individual animal, thus reducing the numbers of animals needed per experiment.

Similarly, in cardiac regeneration experiments where we will surgically transplant cells into the heart, we will need to trace or track their viability, location and observe how they integrate into the tissue over the course of time. With the help of non-invasive *in vivo* imaging methodology, such as echocardiography or MRI, we can monitor what happens to the transplanted cells *in vivo* and perform longitudinal studies (research designed to measure or determine the same variables repeatedly over time) without the need to kill the animals at each time point of the analyses. This means, we can get clear information about the fate of the transplanted cells from the same animal over the course of the experiment, thereby minimizing variability arising from the use of multiple animals.

Fifth, the experimental group sizes will be determined by detailed power calculations based on pre-existing data for likely effect size and chosen to provide the minimum needed for robust statistical analysis. Importantly, we will incorporate longitudinal analysis from non-invasive ultrasound imaging where possible to increase the statistical power (as each animal can act as its own control), ensuring that the experiments do not need to be repeated unnecessarily.

Sixth, when possible, we will use temporary and rapid methods to induce gene expression modifications which will not require the establishment of genetically modified lines and crossing between several genetically modified strains. We have learnt from our collaborator, a method to inject lentiviral particles delivering shRNA (genetic material that can suppress the genes they are designed to target) or transgenes (new genes inserted into a cell) via the jugular vein. When injected into the blood via the jugular vein, these viruses can enter the cells of the blood vessels such as the aorta. This method allows us to modify genes in at specific locations at specific times in the aorta allowing us to study selected processes clearly without having to generate complex genetically modified strains. The establishment of such lines usually requires considerable breeding of multiple genetically modified mouse lines, and our method will considerably reduce the number of animals used for the experiment, possibly up to 90% depending on the complexity of the genetic breeding scheme.

Finally, we will use the PREPARE guidelines prior to initiating any experimental study to aid in the planning of each stage, and the ARRIVE guidelines to help in the design, analysis and reporting of all studies. We will ensure that all experiments are conducted and eventually, the data analysed in a blinded and randomised fashion to minimise bias and only report robust effects. For example, in a typical imaging experiment, the experiment will be conducted and the data analysed by two different technicians and the genotypes and identities of the animals will be masked during data analysis. Equally, cohorts of animals containing test and controls will be subjected to procedures in a randomised manner to ensure consistent and equivalent treatments.

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

We will use efficient breeding strategies (including the use of the NC3Rs breeding and colony management guidelines to support our breeding strategies: Breeding and colony management | NC3Rs) to generate only the required number of animals. We also use the information from pilot studies to determine the number of animals to be used. We also maximise the outputs from each animal by using the tissue for other projects or by sharing with groups who might find them of use within the community.

A retrospective assessment of reduction will be due by 6 October 2031

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.

First, we need to understand the nature of cardiovascular disease. For this, we need to use models that will recapture the causes and the symptoms of the disease. For example, we use genetically modified mice that bear the same mutations (change in their genetic code) as patients who have aneurysms, to see how the disease develops and ultimately manifests. To simulate heart attack conditions, we use rats and tie off or restrict the blood flow in their coronary arteries. This leads to heart muscle damage as with patients who have had a heart attack.

Second, we will need to evaluate treatment options for these diseases. We will use the above-mentioned disease models to treat and evaluate different therapeutic approaches. For example, one new approach that we are investigating to treat heart attacks is by repairing or regenerating the damaged tissue of the heart. If we do that, then we can restore it to full functional capability. To repair heart muscle in the heart attack model, we will physically inject or place a patch or plaster of cells on the damaged area which will replace the lost and damaged cells in that area. The cells would be heart muscle cells to replenish the destroyed muscle supplemented by additional “helper” (epicardial) and “precursor” (endocardial) cells. These are cells that, in the womb, originally built the heart and its blood vessels (cardiac development). This method is a key element of an approach called regenerative medicine.

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Another key area of interest to us is the treatment of aneurysms and other diseases that affect the blood vessels (vascular diseases). These can cause sudden death and are often undiagnosed until a late stage of progression. Even when diagnosed during progression, the condition can be unpredictable and has few treatment options other than major surgery.

Choice of Species:

The mouse is the ideal animal since it is readily amenable to genetic modification and models of injury, vascularisation (blood vessel formation studies), aneurysm and atherosclerosis (plaque formation in blood vessels that obstruct blood flow and cause strokes and heart attacks) are well described. Indeed, genetically modified lines for gene modification specifically in cells of the epicardium and model of aortic aneurysms are readily available. The mouse also breeds quickly and is well established for laboratory research. The experiments on cardiac regeneration however require a rat model since we and others have found that the transplanted human cardiovascular cells do not survive long term in mice – possibly due to differences in heart rate and electromechanical coupling (electrical connection between heart muscle cells that allows to contract and relax synchronously as the heart beats) with the host heart or differences in the immune response. These issues, while present, do not pose as much of a problem in rats and hence these are the preferred rodent models for developing therapies that aim to regenerate failing hearts with human cardiovascular cells. Other species such as rabbits or pigs breed more slowly and are less established than rodents. For most of our experiments we will use juvenile and adult animals for both rats and mice. In some cases, in mice, where we would need to study processes relating to cardiovascular development (how the heart and blood vessels are formed during gestation) or function, we will additionally use pregnant adults, embryos, neonatal and aged animals.

These models are selected to be the most efficient for recapturing the human condition. They are expected to mimic as best as possible the progression of the disease in humans while minimising distress and suffering and avoiding long-lasting harm to the animals. Our methods include strict humane end points where, clinical signs of prolonged distress (usually persistent over 48h) will prompt us to end the experiment and humanely kill the animals. For example, in our severe procedures for inducing a heart attack via open chest surgery or inducing severe aneurysms, we will monitor the animals with non-invasive imaging techniques that will allow us to detect early signs of heart failure or aneurysm rupture. In this way, we will be able to humanely kill the animals before they suffer or experience pain. In some severe aneurysms, the animals will not show signs of distress but experience sudden death due to rupture of the blood vessel. These are hard to predict and occur without warning, but the animals themselves experience only transient suffering. For these models also, our monitoring methods allow us to make the best possible predictions before the animals reach this condition.

We will carefully design each experiment and use the most optimal model - whether genetically modified strains or induced via specific procedures - to model the disease and study the effect of therapies, while making sure the animals experience the minimal amount of distress.

Refined methods for minimal suffering on severe protocols: We have developed strictly regimented protocols and methods to minimise animal distress during and after critical procedures; especially in the protocols of severe category. This has allowed us to generate the best models with least distress and harm to the animals.

Major examples are detailed below:

Myocardial Infarction/heart attack model via surgical coronary artery ligation: This is a severe model that can result in significant animal death. It is critical to understand the factors that affect the response of the heart and its circulation to myocardial infarction and develop therapies. For open-chest surgery in rats to conduct myocardial infarction, we will implement procedures learnt from our collaborators in Seattle, that have enabled them to lower the mortality of the myocardial infarction and cell therapy procedures to between 5-10%. Specifically, we will administer carefully calculated doses of appropriate anaesthetics before the surgery begins and during the surgery. We will perform the surgery with only the minimal tissue handling required for the myocardial infarct (tissue damage by heart attack). This also applies during the cell injections. We will carefully monitor the breathing pattern during the surgery and ensure fast reaction to rectify problems (e.g. atelectasis i.e. collapse of the lungs due to slipping of the breathing intubation). We will carry out surgeries early in the day allowing intensive monitoring (three times) in the afternoon and evening, twice daily over the next 3 days, and daily thereafter to ensure we detect any suffering promptly. Any weight loss is documented and reacted to by having a change in the feeding regimen. Since rodents frequently do not show signs of pain, then we will administer analgesics (pain medication) pre-emptively for 48 hours (or longer if required) without waiting for clear signs. Although, in this procedure a heart attack is induced with localised damage or injury to the heart muscle, we expect the heart to still function i.e. compensated heart failure. Therefore, if the procedure is successful, the animals should not show symptoms and are characteristically well and move freely in the early stages. However, if decompensation i.e. loss of heart function occurs, then there will be clear symptoms including cyanosis (blue coloration of extremities such as toes), oedema (puffy appearance due to fluid accumulation) of the extremities and laboured breathing. Regular observation of the animals allows these symptoms to be used as humane endpoints with immediate humane killing. As with humans, some deaths are sudden and presumed to be arrhythmic (due to irregular heart beats). We have introduced an additional refinement to this procedure by adding an optional step. Instead of performing a second thoracotomy (open chest surgery) to introduce the therapeutic cells or patch of cells to the injured area of the heart, we can, in some experiments, inject the cells percutaneously (from the surface of the skin) using ultrasound imaging to guide us to the site where they need to be e.g. the pericardial space (space between the membranes i.e. the tissue layers covering the heart). This approach is much less invasive than a surgical approach and allows us to continue to refine our cardiac repair strategies; and importantly may be translatable to patients in the future.

Implementing these methods, allows us to streamline the heart attack model. The procedures used with the model will minimise suffering as much as possible to the animals.

Severe Aneurysm model: Some procedures (e.g. severe aneurysm formation) may result in death in a high proportion of animals anywhere from 50-80%, and they therefore have a severe severity classification. These models are used to find targets or test treatments that reduce the formation and rupture of aneurysms. These are complex pathologies that cannot be modelled in vitro. A severe limit may be needed where the control group needs to experience a sufficient percentage of events (e.g. rupture of aneurysms which is when the artery is highly enlarged and the thin walls break) that can be reduced by a treatment, with adequate statistical power (a sufficiently sized experimental group to allow for clear observation of patterns e.g. response to therapies after myocardial infarction) for a definitive conclusion. Experiments with severe limits would only be undertaken after extensive pilot work to demonstrate the mechanisms underlying the pathology (e.g. aneurysm formation), and the efficacy of any proposed treatment in our complex and disease relevant in vitro models. Studies that have ‘hard’ end points in experimental studies (aneurysm rupture, death) provide the confidence

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necessary to move these treatments to human studies, ultimately reducing the numbers of animals required for these studies. In our collaborator's large previous experience with aneurysm rupture studies, 90% of deaths are sudden and could not be expected despite close monitoring twice daily. In 10% of deaths, mortality was preceded in the previous 18 hours by some signs of reduced activity or hunching. However, such signs are not specific, nor predictive of rupture because most animals with such signs recover and do not undergo aneurysm rupture. Nevertheless, if animals show signs of distress, we will monitor them more closely to try to predict those who will ultimately undergo aneurysm rupture. We will also consider doing more non-invasive imaging on those mice (ultrasound) to try to detect imminent signs of aortic rupture.

All procedures will be continually evaluated, reviewed and refined to minimise and reduce experimental duration, animal numbers and suffering while maintaining or improving scientific benefits. To facilitate this, we will have regular meetings within the lab after every series of experiments and we will also have similar discussion with our close collaborators.

Moderate procedures: When the drug tamoxifen is used as a transgene inducing agent, it is mostly administered through intraperitoneal injections. We will use the oral route (in food pellets) only rarely in longer term studies which avoids side effects associated with prolonged oil injection. Food pellets will be refreshed often to ensure maximum palatability. Upon tamoxifen addition to cages, tamoxifen diet will be mashed along with additional food supplements like Nesquik provided daily to make the pellets to make them more palatable. If animals do not readily eat the tamoxifen dosed pellets, then we will revert to oral gavage as the least severe way to introduce tamoxifen. During tamoxifen administration, an observations sheet will be employed to ensure signs other than weight loss are also included in the end point assessments.

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

It is not possible to use non-mammalian models to answer some of the questions we are addressing regarding vascular aneurysm disease and cardiac injuries and therapeutic strategies. While non-mammalian models e.g. zebrafish can provide valuable insight into the molecular mechanisms of cardiac repair, they are not evolutionarily close enough in their cardiovascular system to warrant their use in testing therapeutics. Rodents - mice and rats are the least sentient models that we can utilise to determine the modifiers of vascular disease and cardiac injury and develop good therapeutics for.

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

Animals are housed according to the best recommendations in an appropriate and enriched environment. By performing pilot studies and choosing well established protocols based on our and our collaborators' extensive previous experience, we minimise the unknown effects on the mice and subsequently pain, distress and suffering. In addition, we will ensure there is optimal handling and interaction with the animals to maximise their welfare. For example, we will use non-tail handling methods wherever possible for rodents and will ensure all personnel are trained on this. We will also ensure there is regular interaction and social enrichment for rats, including handling and tickling from a juvenile stage, to promote their welfare (as recommended on https://www.nc3rs.org.uk/news/tickling-rats-social-enrichment-improve-rodent-welfare). In addition, every effort will be made to reduce stress by ensuring familiarity to the handlers as much as possible.

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We and others have extensively refined every procedure we use. For all severe protocols, we routinely monitor the animals closely to ensure they are not in distress or experiencing sudden and adverse effects from any of the procedures. For example, animals that are susceptible to severe and fast progressing aneurysms (Marfans syndrome model), are imaged via echocardiography, at least once a week to monitor the progression of the aneurysm. We established an earlier time point here - when the aorta diameter reaches 2.8mm (approximately twice the size of a normal mouse), we will humanely kill the animal and end the experiment. This minimises any suffering.

Where high salt or high fat diet are used and animals suffer skin irritations, we will implement more frequent cage cleaning, say once a week to reduce the risk of skin irritation, trimming the tips of the hind claws, before and during the provision of the altered diet. These would help to minimise the risk of skin lesions occurring. We will also consider including the use of topical treatments, as advised by the NVS, to provide further relief to the animals that are affected.

Similarly for cardiac injury models, we have instituted extensive post-operative care and monitoring regimes to minimise distress and institute humane end points. We follow well-validated models and highly refined procedures for these models.

For treatment of animals with tamoxifen as a transgene modulating agent, we will first acclimatise them to their new diet by incorporating mashed food pellets supplemented with products like Nesquik. After this, tamoxifen will be included in the mashed food and provided daily. We will also follow the internal NVS guidance on Tamoxifen usage to direct us.

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

We use published guidelines to assist with planning animal research and testing, such as the PREPARE guidelines: http://journals.sagepub.com/doi/full/10.1177/0023677217724823 and the ARRIVE guidelines https://arriveguidelines.org/arrive-guidelines. We also use other resources including guidance and publications from the NC3Rs and Laboratory Animal Science Association.

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

We will regularly check information on NC3Rs website, we’ve signed up to the NC3Rs newsletter through our institution. We regularly attend Regional 3Rs symposia and other local webinars provided through our establishment. We are part of crucial mailing lists that allow us to share animals and materials to maximise the output from each animal. We have also joined the NC3Rs Cardiovascular network: https://nc3rs.org.uk/3rs-resource-library/cardiovascular-network. We have access to the Named Information Officer here as an invaluable resource of 3Rs information.

A retrospective assessment of refinement will be due by 6 October 2031

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?

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